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BLOOD GROUP A₁

A POSSIBLE SUBDIVISION INTO TWO TYPES, PROBABLY HEREDITABLE [1]

by

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(Received for publication December 27, 1956)

In 1954 G. W. G. Bird (1) published a paper in which he describes reactions of seed agglutinins with panagglutinable erythrocytes. The cells had been »transformed» by a T-receptor unmasking fluid occasionally found in a bacterially contaminated blood sample. From his experiments with extracts of *Dolichos biflorus* (anti-A₁), *Phaseolus lunatus* (anti-A) and *Phaseolus vulgaris* (unspecific in respect to ABO-blood groups) he concluded that some seed extracts with well-defined anti-A specifity against untreated cells, s.a. *Phaseolus lunatus*, seemed to be able to detect the T-receptors, whilst others, s.a. *Dolichos biflorus*, failed to do so.

We (2) have repeated these experiments with a larger scale of blood-group specific phytagglutinins intensively studied (3): anti-H from *Laburnum alpinum, Cytisus sessilifolius, Lotus tetragonolobus;* anti-A from *Vicia cracca, Phaseolus lunatus, Dolichos biflorus;* anti-B+A from *Sophora japonica, Coronilla varia;* anti-B+H from *Evonymus europaea, Marasmius oreades.* For the transformation of red cells, i.e. for rendering them panagglutinable, we used a highly efficient T-ferment preparation obtained as filtrate from a water-Vibrio strain selected among more than 20 strains [2]; for some

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[1] Preliminary report investigations with »T-ferment«treated red cells and plant agglutinins.

[2] We are indebted to Dr. Caselitz, Tropeninstitut Hamburg, and Dr. Brandis, Hygiene-Institut Frankfurt/M, for their kindnesses in supplying us with the different strains of water-Vibrios, partly preselected for T-transformation.

instances we used also suspensions of *Myxovirus influenzae* type A (FM1). Moreover, we compared the effects of these transforming reagents with the changes produced by some proteolytic enzymes, s.a. trypsin, papain and ficin.

From our experiments (titrations, absorptions, inhibitions) we believe that blood group specific plant extracts — so far as included in our investigations — never contain any traces of T-agglutinins. But we found, that by the action of T-ferment there occur very much interesting changes on the surface of the red cells concerning the specific ABH-substances. The results of our studies will be reported in details at another place.

Here, however, we like to deal with our observations on the effect of T-transformation caused on red cells from persons belonging to A_1 or A_1B group in respect to their different behaviour against anti-H agglutinins from plants and eel-serum.

MATERIALS AND TECHNIQUES

1. Anti-H Agglutinins. — a) Extracts from seeds of *Laburnum alpinum* and *Lotus tetragonolobus*. These were prepared in the usual manner as firstly described by Renkonen (4). The saline-titers of the obtained extracts were 1 : 16—32 against normal O-cells, 1 : 8—16 against A_2-, 1 : 2—4 against B- and only tr. — if occurred — against A_2B-cells. The extracts failed completely to agglutinate normal A_1- or A_1B-cells.

b) Eel-serum [1] with a titer in saline of 1 : 32 against O-, 1 : 16—32 against A_2- and 1 : 2 against B-cells. It also never reacts with normal A_1- or A_1B-cells.

2. Red Blood Cells. — Mostly freshly drawn blood specimens were used; but no changes in the marked reaction could be observed when aged up to 3 days kept at low temperatures.

3. Preparations of T-Ferment. — a) Water-vibrio strain No. 162 found the best producer of efficient T-ferment was used for all transformations. It was cultured in peptone water (1 per cent) at $p_H = 7.2$ for 48 hrs. at 37° C and further 48 hrs. at room temperature, finally filtered through a Seitz-filter. This sterile filtrate was used as transforming reagent without further treatment; it

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[1] a selected one out of several eel-sera supplied by the courtesy of Dr. Dickgiesser from the »Behringwerke Marburg« Germany.

remained active with no remarkable loss for a period of more than 2 months if kept in the icebox. Its capacity as virus-receptor destroying enzyme (RDE) was established by controlling the non-agglutinability of transformed red cells by the known hemagglutinating power of virus suspensions from *Myxovirus influenzae.*

b) *Myxovirus Influenzae.* In a few instances we transformed with allantois fluid from hen-embryos infected with influenza virus type A (FM1) containing active viruses, which had a hemagglutinating titer of 1 : 128 against human cells. The activity of this fluid was observed to be unchanged after a period of 3 months if kept in the icebox.

4. T-Transformation of Red Cells. — a) Vibrio filtrate. 0.1 ml of twice washed packed red cells were incubated at room temperature (18—22°C) with 2.0 ml of the filtrate for 5—30 min. Controls of the occurrence of transformation were performed in slide tests by adding one drop of A- resp. AB-serum to one drop of the red cell suspensions in the Vibrio filtrate. Panagglutinability happened in the earliest after 3—4 min., then increased up to its maximum after 20—30 min. Then the filtrate-suspensions of the red cells were twice washed with saline to free them off the peptone water, the packed red cells resuspended in saline preparing a 2—3 per cent suspension. Controls of red cells treated in the same manner with peptone water not containing T-ferment showed no reactions neither with T-agglutinins in normal human sera nor with anti-H phytagglutinins nor with eel-serum.

b) Influenza Virus type A (FM1). 0.1 ml of twice washed packed red cells were mixed with 1.0 ml of the virus suspension. This mixture was gently shaked and allowed to stand at room temperature for 1 hr, then incubated at 37°C for 1—2 hrs. After this time the viruses had liberated from the formerly attached erythrocytes, i.e. desagglutination had taken place. The suspensions of free viruses an red cells were now twice washed with saline and the packed red cells resuspended in saline to 2—3 per cent.

RESULTS

At the beginning of our studies it seemed that we could agree with Bird in assuming that there exist T-agglutinins containing and not containing extracts from plants with ABH-blood group

specifity. But soon it became evident, that in the case of apparently
»unspecific» reactions T-agglutinins do not play any role. We
observed that these unspecific reactions did occur mainly with
some of the well-known anti-H or anti-B+H specific plant extracts
if transformed red cells were used for agglutination tests. Trying,
for instance, with the seed extracts from *Laburnum alpinum* all
of the examined A_1- or A_1B-cells were strongly agglutinated in the
slide test, unlike the behaviour of these extracts against untreated
A_1- or A_1B-cells. However, titrating the Laburnum-extracts we
could establish, that O-, A_2- and B-cells had slightly more elevated
titers than A_1- or A_1B-cells (see table 1). Furthermore, transformed
A_1- or A_1B-cells used for absorption tests could diminish the specific
reactivity of the Laburnum extracts against untreated O-cells to
a markedly higher degree than do normal A_1- or A_1B-cells. Twice
repeated absorptions resulted in exhausting completely the aggluti-
nating power for normal O-cells, and the titers against transformed
O-, A_2- and B-cells decreased markedly. The hemagglutination of
transformed A_1- or A_1B-cells by the Laburnum extracts could be
inhibited specifically by human H-substances (crude or purified)
and by salicin, as described typical for the anti-H agglutinins from
Laburnum alpinum by one of us previously (3). These findings do
not agree with the behaviour of T-agglutinins in normal human
sera. These, in contrast, can be separated by absorption from the
blood group specific agglutinins anti-A or anti-B and cannot be
inhibited by any blood group substances.

The reactions with seed extracts from *Cytisus sessilifolius* were
quite the same as with the extracts from Laburnum alpinum.

The anti-B+H active extracts from *Evonymus europaea* and
Marasmius oreades showed no differences in the titers against
transformed red cells of all ABO-groups, but — like Laburnum
alpinum and Cytisus sessilifolius — the specific reaction with nor-
mal B- and O-cells could be absorbed by transformed A_1-cells;
furthermore, the reaction with transformed A_1-cells could be inhib-
ited by soluble B-and H-substances.

In contrast to the above described behaviour of the anti-H
phytagglutinins the seed extracts from *Lotus tetragonolobus*, sur-
prisingly, did not react with each individual sample of blood group
A_1 or A_1B after being transformed: some specimens were aggluti-
nated strongly, others failed completely to be agglutinated. In this

TABLE 1

TITERS [1] OF A SEED EXTRACT FROM *Laburnum alpinum* AGAINST BLOOD CELLS

Dilu-tions	a) Untreated						b) T-Transformed					
	O	A$_2$	B	A$_1$	A$_1$B	A$_2$B	O	A$_2$	B	A$_1$	A$_1$B	A$_2$B
undil.	+ + +	+ + +	+ +	—	—	±	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
1: 2	+ + +	+ + +	+	—	—	—	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
4	+ + +	+ + +	+	—	—	—	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
8	+ +	+	—	—	—	—	+ + +	+ + +	+ + +	+ + +	+ +	+ + +
16	+	—	—	—	—	—	+ + +	+ + +	+ + +	+ +	+	+ +
32							+ + +	+ +	+ +	+	—	+
64							+ +	+	+	—	—	—
128							+	—	—	—	—	—

[1] Centrifugation method.

TABLE 2

TITERS [1] AGAINST TRANSFORMED RED CELLS WITH

Dilu-tions	a) Anti-H in Seed Extract from *Lotus tetragonolobus*						b) Anti-H in *Eel-Serum*					
	A$_1$ (Pfei.)	A$_1$ (Berg.)	O	A$_2$	B	A$_1$B	A$_1$ (Pfei.)	A$_1$ (Berg.)	O	A$_2$	B	A$_1$B
undil.	+ + +	—	+ + +	+ + +	+ + +	—	+ + +	—	+ + +	+ + +	+ + +	—
1: 2	+ + +	—	+ + +	+ + +	+ + +	—	+ + +	—	+ + +	+ + +	+ + +	—
4	+ + +	—	+ + +	+ + +	+ + +	—	+ + +	—	+ + +	+ + +	+ + +	—
8	+ +	—	+ + +	+ + +	+ + +	—	+ + +	—	+ + +	+ + +	+ +	—
16	+	—	+ + +	+ + +	+ +	—	+ +	—	+ + +	+ + +	+	—
32	—	—	+ + +	+ + +	+	—	+	—	+ + +	+ + +	—	—
64	—	—	+ +	+ +	—	—	—	—	+ + +	+ + +	—	—
128	—	—	+	+	—	—	—	—	+ +	+ +	—	—
256	—	—	—	—	—	—	—	—	+	+	—	—

c) T-Agglutinins in *AB-Serum*			d) Anti-A$_1$ in *Dolichos biflorus*				
1:16—32			1:512	1:512	—	1:16	—	1:128

[1] Centrifugation method.

TABLE 3

AGGLUTINATION-REACTIONS OF BLOOD CELLS FROM UNSELECTED PERSONS BELONGING TO:

With Anti-H	A$_1$-Blood Group			A$_1$B-Blood Group		
	Nr	Positive	Negative	Nr	Positive	Negative
a) from *Lotus tetragon.*	73	17	56	4	1	3
b) in *Eel-serum*		17	56		1	3

respect too, the known difference between the anti-H agglutinins from *Laburnum alpinum* and *Cytisus sessilifolius* on the one side and from *Lotus tetragonolobus* on the other side — firstly established by Morgan and Watkins (3) and later confirmed by one of us (3) in respect to their different affinity to native H-substances and simple sugars — becomes evident once more. Therefore, because the anti-H agglutinins from eel-sera have been found to have a closer relationship to the Lotus-anti-H than to the Laburnum-anti-H, we used eel-serum too. The reactions of the eel-serum were fully in accordance with those of the Lotus-extract, i.e. the same samples of A_1- or A_1B-blood cells, which had been agglutinated by the Lotus-agglutinins, were also agglutinated by the eel-serum, whilst the samples negative with Lotus-extract were also quite negative with eel-serum (see table 2). This behaviour of the eel-serum indicates that it do not contain T-agglutinins for human transformed red cells.

Now, we have examined 73 samples of A_1- and 4 samples of A_1B-blood cells unselected for learning something about the frequences of these two different A_1-subtypes, both with the seed extract of *Lotus tetragonolobus* and eel-serum. The results of this investigation is presented in table 3.

As it can be seen from this table the reacting type of A_1-cells — we called it the »Pfeiffer-type»[1] — has a frequence in our unselected material of about 23 per cent. The non-reacting type was called by us the »Berger-type».[1] In A_1B cases this subtype is recognizable too. Repeated tests of blood cells from the same A_1 persons — both negative and positive — gave constantly the same distinct results. The reactions with the agglutinins from *Lotus tetragonolobus* and eel-serum were always in accordance.

Now, we examined in a briefly informative manner related persons possessing the A_1 blood group. In the family of our technician, Mr. Christa Pfeiffer, we established the reacting type at her mother and at her sister. In three families we found the non-reacting A_1-type at 3 of the parents and at all four of their A_1-children. In one case of disputed paternity the A_1-type of the mother differed from that of the child; but the non-reacting type of the child was in agreement with the type of that man, who could not be excluded,

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[1] Names of our technicians, where these two different A_1-subtypes were discovered at first.

whilst the second suspicious man could be excluded by other blood group-systems.

The hemagglutination of the transformed A_1- or A_1B-cells of the reacting »Pfeiffer-type» with Lotus-agglutinins or agglutinins from the eel is inhibited specifically by H-substances and L-fucose. Absorptions of the reacting agglutinins in both reagents with suitable amounts of transformed reacting A_1-cells were also able to exhaust the agglutinating power against normal O-cells. The »Berger A_1-type», however, failed to do so.

The two distinguishable A_1-types could not be differentiated, neither by the anti-A_1 agglutinins in extracts from *Dolichos biflorus* (see table 2) nor by anti-A_1 in absorbed animal sera. Any relationship of the anti-H sensitivity in the reacting A_1-types after being transformed with other blood group systems could not be detected, especially not in connection with the Lewis-blood group system; thus, two Le^a positive A_1 persons belong to different A_1 subtypes. We d'ont believe, that the reacting »Pfeiffer-type» is identical with the so-called »A intermedius».

DISCUSSION

Our knowledge about the nature and action of the »T-ferment» produced by a variety of bacteria and viruses is yet now incomplete in many respects. There are some authors, which ascribe to it a »mucinase-like» activity. The interest in this enzymatic substance is focussed mainly to the relation between the »T-agglutinins» in normal sera from many animals and the »T-agglutinogens (-receptors)» which are believed to be unmasked on the red cell surfaces by its action. Our presented findings seem to suggest, that there is not only the narrowly limited action on the virus-receptors — estimated as its specific enzyme-substrate — but there must be in addition some influence on the ABH-antigens on the red cell surfaces. Perhaps, the T-ferment may cause some hydrolytical effects concerning the terminal molecule-groups on the macro-molecules of the ABH-substances. Thus, it seems to us, that the H-substance, inherent in all ABH-antigens, is more strongly affected than the A or B substances. The anti-H agglutinins from *Laburnum alpinum* are known to possess a great affinity to the native H-substance but no to L-fucose, while those from *Lotus*

tetragonolobus have a more less affinity to the native H-substance but a pronounced reactivity with L-fucose. Eel-serum in addition reacts strongly both with the native H-substance and L-fucose. Therefore, we decline to the conclusion, that the differences between the two described A_1-types, recognizable by the action of T-ferment, may be evocated by different structures in the overall occurring H-substances in respect to liberate more or less L-fucose units as terminal serologically reacting groups on the surfaces of the macro-molecules, which is effected by the T-ferment.

Findings not here presented in our studies (2) with one strongly specifical anti-A extract from *Phaseolus lunatus* (if using normal cells) seem to support this view of a different manner in unmasking terminal groups on the ABH-antigens by the action of T-ferment. These anti-A agglutinins reacted, if only in a low degree, with transformed B-cells but not with transformed O-cells. This reaction could not be absorbed by normal B- or transformed O-cells but only by transformed B-cells and, of course, by normal and trans-formed A_1-cells. D-galactose was not able to inhibit this reaction with B-cells. Furthermore, red cells from hens are not agglutinated by blood group specific plant agglutinins. If we use T-ferment transformed hen's cells they are now agglutinated in a specifically-like manner only by anti-B active plant agglutinins and agglutinins from *Soja hispida*, known to react not with human B but with rabbit's cells. This reaction can be inhibited specifically by D-ga-lactose. Aside from this reaction with anti-B agglutinins we observe too a strong reaction of the transformed hen's cells with extracts from *Phaseolus lunatus*, which could not be inhibited by D-galac-tose.

Further detailed studies about these phenomenons are neces-sary. It may be possible that in the case of the A_1-bloodgroup other more gradual differences can be observed.

The differentiation of two probably heriditable subtypes of the A_1 blood group can serve as a supportant determination of the individual A_1-property, comparable with the value of the S- and s-antigens in M/N-groupings.

The results of our studies with T-ferment transformed red cells and plant agglutinins demonstrate once more that the blood group specific plant extracts contain proteins with one serological specifity only not mixed with other globulins with different serological

specifities, as it is the case in animal sera, normal or immune. Therefore, they are — in our opinion — reagents well appropriated for serological analysis concerning the ABH-substances. In addition, they show that using enzymes as adjuvants for revealing hidden antigens on red cells or antibodies this method, in some instances, do complicate more than simplify the interpretions of observed agglutination phenomenons.

SUMMARY

Using blood cells being transformed with T-ferment for hemagglutination tests with anti-H agglutinins in seed extracts from *Lotus tetragonolobus* or in eel sera it is possible to distinguish two different types of the blood group A_1 in man: one which is agglutinated and one which is not agglutinated.

The reacting A_1-type occurred in a frequence of about 23 per cent in our unselected material (77 blood samples). In A_1B cells these subtypes are recognizable too. They seem to be heriditable.

The reaction with the positive reacting A_1-cells can be inhibited by the H-blood group substance and L-fucose, indicating that T-ferment do not act only on the surfaces of red cells by unmasking the »T-receptor» but, perhaps, too by unmasking the H-substances inheritent in all blood groups, but, apparently, in a different manner.

REFERENCES

1. BIRD, G. W. G.: J. Path. Bact. 1954:*68*:289.
2. KRÜPE, M., OSTERMANN, P., DÖTZER, W., and MAUS, H. G.: in preparation.
3. KRÜPE, M.; see his book: »Blutgruppenspezifische Eiweisskörper (Phytagglutinine)» Ferd. Enke Verl., Stuttgart 1956.
4. RENKONEN, K. O.: Ann. med. exp. biol. Fenn. 1948:*26*:66.

FROM CHILDREN'S CLINIC AND THE DEPARTMENT OF SEROLOGY AND
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THE WATER CONTENT OF THE ALCOHOLIC PRECIPITATE
OF NORMAL AND RHEUMATIC SERA

by

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Jokinen (1) has described a serum test for the diagnosis of rheumatic diseases which he found to be positive in 71% of cases. In this test serum proteins are precipitated in 94% ethanol, after which the precipitate is centrifuged off and subjected to the action of conc. sulphuric acid. If a brown colour develops and the mixture warms up, the reaction is considered positive. The writer suggests that the behaviour of the precipitate in sulphuric acid might be governed by the water and alcohol bound to the precipitate.

As the explanation appeared probable, this study was undertaken to discover whether the mode of reaction was influenced by the water content of the precipitate.

TECHNIQUE

The technique described by Jokinen (1) was followed as closely as possible with the only exception that the amount of serum was raised to 1 ml and that of the ethanol correspondingly to 10 ml. Although the precipitation technique is rather unsuitable for accurate·water determination, it was used in order to obtain results which could be compared with those given by the precipitation test of Jokinen.

10 ml of 94% ethyl alcohol was measured into a test tube of inner diameter 11—12 mm. 1 ml of serum was pipetted into it at

room temperature without the drops touching the sides of the tube. The tube was placed, without shaking, in a refrigerator at +4°C, where it was allowed to stand unsealed for 24 hours. It was then centrifuged at 2500 r.p.m. for 5 min. in an angle head centrifuge. The supernatant was discarded and the tube inverted and allowed to stand at room temperature for 15 min. 10 ml of double distilled water-free methanol was now added to the precipitate, the mixture was stirred vigorously, and heated in a water-bath to the boiling point of the mixture, using a capillary tubing to avoid excess loss of methanol. The tube was now sealed with rubber stopper and stored for 4 days in a refrigerator. After this the mixture was centrifuged for 5 min. at 2500 r.p.m. and the supernatant collected and shaken. From the supernatant a sample of 1 ml was taken, the water content of which was determined by the Karl Fischer water-titrating method (2). The assays were run in duplicate. The results are given as mg of water bound to the precipitate obtained from 1 ml of serum.

Alongside the water determinations, the original Jokinen test was performed on all the sera studied. The result was considered negative (—) when the precipitate remained completely undissolved or gave a faint brownish colour. The result was taken as weakly positive (±) if some of the precipitate clearly dissolved and a weak brown colour developed, and strongly positive (+) if the precipitate was completely dissolved and a strong brown colour developed.

MATERIAL

The material consisted of 47 sera obtained from adult hospital patients. 28 of them had rheumatoid arthritis and 19 rheumatic fever. [1] As a control series, the sera of 17 unselected blood donors were examined. [2]

RESULTS

The correlation between the amount of water bound to the precipitated protein and the erythrocyte sedimentation rate (ESR) can be seen from Table 1. When comparing the different groups ar-

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[1] The samples were from the Medical Clinic III of the University of Helsinki.

[2] The sera came from the blood bank of the Finnish Red Cross.

TABLE 1

Case No.	Diagnosis RF-Rheumatic Fever RA-Rheumatoid Arthritis	ESR	Water in mg Bound to the Final Precipitate Classed According to ESR				Jokinen Test
			—20	21—50	51—100	101—	
1	RF	8	141				—
2	RF	8	147.5				—
3	RF	11	141				—
4	RA	13	152				—
5	RF	15	147				—
6	RF	15	157.5				—
7	RF	16	142.5				—
8	RF	16	157				—
9	RA	16	164				—
10	RA	16	175				—
11	RA	17	165				—
12	RF	18	150.5				—
13	RA	18	178				—
14	RF	19	146				—
15	RF	19	155				—
16	RF	20	164				—
17	RA	21		148			—
18	RA	21		165.5			—
19	RF	21		166			—
20	RF	22		156			—
21	RF	22		175			±
22	RA	22		179.5			±
23	RA	23		166.5			—
24	RA	28		176			—
25	RA	30		156.5			±
26	RA	30		176.5			±
27	RA	34		160.5			—
28	RF	34		170.5			±
29	RF	35		147.5			—
30	RA	44		163.5			±
31	RA	44		180			+
32	RF	50		189			—
33	RA	51			160		—
34	RF	51			176.5		±
35	RF	52			178		—
36	RA	52			202.5		+
37	RA	53			177.5		±
38	RF	58			178		±
39	RA	63			184		+

Cont.

Case No.	Diagnosis RF-Rheumatic Fever RA-Rheumatoid Arthritis	ESR	Water in mg Bound to to the Final Precipitate Classed According to ESR				Jokinen Test
			—20	21—50	51—100	101—	
40	RA	65			172		±
41	RA	66			176.5		±
42	RA	70			170		—
43	RA	82			184.5		+
44	RA	98			197		±
45	RA	111				204	+
46	RA	115				190	+
47	RA	126				213.5	+
		Mean	155.2	167.3	179.7	202.7	

ranged according to the ESR a trend to a greater amount of bound water can be recognized in sera from blood possessing an elevated ESR. In the control group of normal sera the amount of bound water is low, as is seen in Table 2.

TABLE 2

Case No.	Water in mg Bound to the Final Precipit.	Case No.	Water in mg Bound to the Final Precipit.
1	157.5	10	120.5
2	157.5	11	161.5
3	153.5	12	179
4	133	13	146.5
5	165	14	159
6	179	15	150.5
7	134	16	171
8	142.5	17	146.5
9	141.5		
		Mean	152.8

As a comparison, the Jokinen test was performed on all the sera tested. In the control group of normal sera and in that where the ESR was ≲ 20 all sera reacted negatively, positive reactions being the more frequent the higher the ESR and the greater the amount of bound water.

DISCUSSION

In evaluating the experimental results, one must bear in mind
the factors affecting the formation of the final protein precipitate.
It must be assumed that, for example, the rate of dropping the
serum into the alcohol, the centrifugation procedure and especially
the drying phase lasting for 15 minutes affect the water content
of the precipitate and thus lead to heterogeneity of the material.
The method for titrating the bound water is in itself an accurate
one.

As to the factors influencing the water content of the precipitate,
nothing definite can be said. Jokinen and Kaipainen (3) and Holo-
painen and Koskinen (4) have measured the albumin/globulin
ratio of rheumatic sera and found that it is usually smaller in sera
giving a positive Jokinen reaction than in normal sera. It might
be assumed that the alterations in the proteins or possibly also in
the composition of other blood constituents would increase the
hydration of the precipitate. It is likely that at the moment when
the serum droplet touches the alcohol layer and the denaturation
of the proteins takes place, certain physicochemical surface reactions
lead to the trapping of water in interspaces of sizes varying in
accordance with the composition of the serum. An another possi-
bility is that the hydration value of the proteins themselves in
rheumatic serum would be greater than in normal serum. Pre-
liminary experiments in which lyophilized normal and rheumatic
sera were subjected to the action of different vapour pressures
of water and the water adsorption measured did not, however,
verify this hypothesis. The amount of water bound to the preci-
pitate appreciably exceeds the maximum hydration value of
the proteins. Thus, for example, if 1 ml of serum contains about
75 mg of protein, it could bind $\lessgtr$ 75 mg of water when maxim-
ally hydrated (5). The amounts of water bound to the precipitates
are, however, much greater, suggesting that it is somehow trapped
in the structure of the precipitate.

SUMMARY

The water content of the protein precipitates from normal and rheumatic sera obtained by a modification of Jokinen's method has been measured. It was found that with increasing ESR the amount of water bound to the precipitate generally increased. Further, the Jokinen precipitation test frequently became more positive with rising water content.

The technique used and the mode of binding water in the protein precipitate are discussed.

REFERENCES

1. JOKINEN, E. J.: Ann. med. exp. et biol. Fenn. 1954: Suppl. No. 5: 32.
2. MITCHELL, J. Jr., and SMITH, D. M.: Aquametry p. 180. New York 1948.
3. JOKINEN, E. J., and KAIPAINEN, W. J.: Ann. med. exp. et biol. Fenn. 1953:31:155.
4. HOLOPAINEN, T. E., and KOSKINEN, H. M.: Ann. med. exp. et biol. Fenn. 1954:32:419.
5. EDSALL, J. T.: The Proteins. p. 562. New York. 1953.

RELATIONSHIP BETWEEN BUFFER CAPACITY AND PH IN THE BLOOD OF SURGICAL PATIENTS

by

S. J. VIIKARI and P. SALENIUS

In modern fluid balance control, increasing attention is being paid to the variation of the blood *pH* during anaesthesia and post-operatively. The *pH* of the blood generally remains fairly constant, but the changes it undergoes reflect the variations in the acid-base equilibrium within the organism. The *pH* level is determined by the buffer system of the blood and the liberation of carbon dioxide and fixed acids by the kidneys.

In the present study the variation of the blood *pH* has been followed and compared with the simultaneous changes in the buffer capacity in connection with various surgical operations.

METHODS

The *pH* of the blood was measured at 37°C under anaerobic conditions using the Astrup apparatus to which a water thermostat was connected. All the determinations were made immediately after the samples were taken. The potentiometer employed was battery-operated, and the *pH* values could be read to the nearest 0.002 *pH*-unit. The buffer capacity of the blood was determined simultaneously at the same temperature. The procedure was that used previously by one of us (2, 3). The determinations were in all cases made on two samples taken at the same time.

MATERIAL

The blood samples were obtained from 15 surgical patients who were preoperatively in good condition. Parallel *pH* and buffer capacity determinations were made on a total of 69 blood samples. For comparison, the hemoglobin concentrations of the blood were also measured. The variation of these values was followed over periods from 4 to 21 days during the postoperative stage. In all the patients the function of the kidneys was observed to be normal.

RESULTS AND DISCUSSION

The subjects of this study are presented in Table 1 in which also the type of operation is given. In the selection of the subjects an attempt was made to obtain data relating to various surgical measures.

An examination of Table 1 reveals that the *pH* of the blood has in most cases remained fairly constant during the postoperative stage. However, when the *pH* has undergone a change, this has always involved a shift to a higher (more alkaline) value. In ten cases the shift in the *pH* has been very slight, but in five cases (cases 4, 10, 11, 13, 14) a more pronounced shift was observed. In all cases the *pH* values tended to revert during the postoperative stage to the levels at which they were prior to the operations.

The buffer capacity of the blood decreased in all cases following the operation despite the fact that the fluid balance was maintained by blood transfusions or fluid infusions. In those cases in which the greatest decreases in buffer capacity were noted, also the *pH* value of the blood underwent a clear increase. This was especially true for cases 11 and 13.

Case 11 (Fig. 1) was a patient on whom lobectomy for pulmonary tuberculosis was performed. On the third day following the operation the buffer capacity of the blood was found to have decreased from 0.0345 to 0.0244. The *pH* had increased from 7.43 to 7.52 during the same period. On the fifteenth day after the operation, the buffer capacity had increased close to the value before the operation, and was 0.0303, and also the *pH* had fallen to the original level. After this period the patient developed a bronchial fistula and his buffer capacity decreased to the value 0.0238. At the same

· TABLE 1

VARIATION OF BLOOD PH, BUFFER CAPACITY ($\times 10^2$) AND HEMOGLOBIN CONTENT
(G/100 ml) DURING POSTOPERATIVE PERIODS

Case	Age and sex	Operation	Value prior to operation	Postoperative values							Length of postoperative period during which blood samples were taken
1.	52 y. male	Cholecystectomy	7.42	7.45	7.42	7.43	7.39				6 days
			3.70	3.57	3.13	3.03	3.33				
			16.6	15.5	12.9	12.9					
2.	50 y. female	Laparotomy	7.36	7.35	7.37	7.37					»
			3.33	3.23	3.13	3.13					
			10.5	10.9	12.3	12.0					
3.	26 y. male	Partial gastrectomy	7.37	7.37	7.38						8 days
			3.70	3.45	3.13						
			16.0	15.0	14.5						
4.	29 y. male	Partial gastrectomy	7.34	7.37	7.42						4 days
			3.70	3.57	3.13						
			15.5	15.5	12.7						
5.	27 y. female	Pneumectomy	7.38	7.39	7.42	7.42	7.41				8 days
			3.13	2.78	2.86	2.86	2.86				
			10.9	9.3	8.4	8.9	9.3				
6.	52 y. male	Exploratory thoracotomy	7.43	7.46	7.43	7.43					6 days
			3.45	3.13	3.13	3.13					
			13.7	12.9	11.6						
7.	43 y. female	Radical mastectomy	7.36	7.41	7.41	7.38	7.38	7.42			8 days
			3.45	3.13	2.86	3.03	3.03	2.94			
			14.1	12.6	10.5		11.2				
8.	53 y. male	Lobectomy	7.41	7.41	7.43	7.45					10 days
			3.33	3.33	3.13	3.03					
			12.9	11.8	12.3						
9.	45 y. female	Cholecystectomy	7.36	7.40	7.41	7.40					5 days
			3.33	3.13	3.03	3.23					
			12.9	12.0	12.0	11.0					
10.	57 y. male	Radical repair of diaphrag. hernia	7.32	7.44	7.39	7.40	7.36				21 days
			3.45	2.70	3.23	3.03	3.45				
			16.0	13.0	15.0	13.2					
11.	34 y. male	Lobectomy	7.43	7.42	7.52	7.49	7.48	7.46	7.42	7.49	17 days
			3.45	3.33	2.44	2.44	2.63	2.63	3.03	2.38	
			15.5	15.5	8.9	8.0	9.3	10.9	11.2		
12.	52 y. male	Sympathectomy	7.38	7.42	7.36	7.41	7.41				8 days
			3.57	2.78	3.03	2.78	3.13				
			12.6	11.2	11.6						
13.	44 y. male	Lobectomy	7.37	7.37	7.39	7.44	7.45	7.49			14 days
			3.57	3.13	2.78	2.70	2.78	2.33			
			15.0	12.0	10.2		10.5	8.6			
14.	56 y. male	Partial gastrectomy	7.32	7.40	7.33	7.35					9 days
			3.45	3.13	3.45	3.45					
			13.7	13.7	12.9	11.0					
15.	38 y. female	Thoracotomy for mediastinal tumour	7.40	7.40	7.43						5 days
			3.23	3.57	3.13						
			13.7	15.0	13.7						

The observations have been grouped so that the upper figure gives the measured pH, the next figure the buffer capacity ($\times 10^2$) at the same time, and the lower figure the corresponding hemoglobin content (g/100 ml).
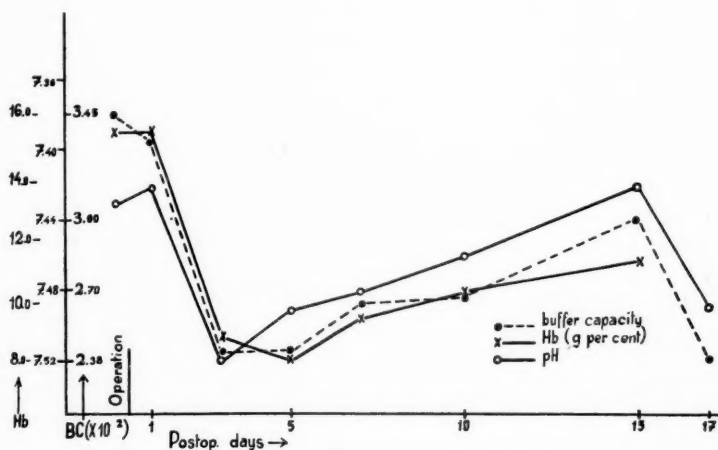
Fig. 1. — Case 11. Lobectomy for pulmonary tuberculosis. As the buffer capacity of the blood decreases, the blood *pH* increases and the hemoglobin content decreases.
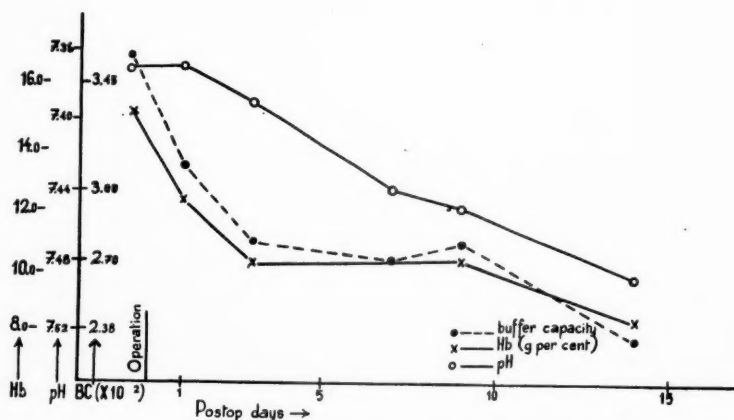
Fig. 2. — Case 13. Lobectomy for pulmonary tuberculosis. Similarly as in case 11, the *pH* of the blood rises as the buffer capacity decreases and the hemoglobin content parallels the decrease in buffer capacity.

time the blood *pH* again shifted in the alkaline direction, as shown by the value for the sample taken on the seventeenth day.

Case 13 (Fig. 2) was also a patient on whom lobectomy for pulmonary tuberculosis was performed. In this subject the decrease in the buffer capacity did not take place as rapidly as in the preceding case, and also the shift of the *pH* level was slower.

As seen in Figs. 1 and 2 and in Table 1, the changes in the buffer capacity paralleled the changes in the hemoglobin content during the postoperative stage; both values decreased. This is natural if view of the known fact that hemoglobin is largely responsible for the buffer capacity of the blood (1).

On the basis of the results of the present study, it is not possible to give the reason for the shift of the *pH* in the alkaline direction as the buffer capacity of the blood decreases. The findings do, however, suggest that if the buffer capacity decreases to relatively low values during the postoperative period, the result will more likely be alkalemia than acidemia.

SUMMARY

Repeated determinations of blood *pH* and buffer capacity have been made on a series of 15 surgical patients during the postoperative period. When a decrease to relatively low values occurred in the buffer capacity, the *pH* of the blood shifted in the alkaline direction.

REFERENCES

1. KILPI, S.: Acta chem. Scandinav. 1952:*6*:475.
2. VIIKARI, S. J.: Acta chir. Scandinav. 1954:*107*:525.
3. VIIKARI, S. J., and KLOSSNER, O.: Acta chir. Scand. In press.

EFFECT OF INFUSIONS ON THE BUFFER CAPACITY OF BLOOD

by

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The buffer capacity of the blood has been found to be primarily determined by the hemoglobin concentration (1). Owing to this, blood transfusions are of great importance in the postoperative period, especially in anemic patients (3). By infusion of glycylglycine (3.74 per cent aqueous solution) it has been found possible to maintain the blood buffer capacity during the postoperative period at the level prior to the operation (4).

The purpose of the present study was to compare the effects of glycylglycine, and macrodex infusions and blood transfusions on the buffer properties of the blood. At the same time attempts were made to determine the component or components of the blood on which the infused solutions have the greatest influence.

The subjects on whom the experiments were performed were patients under treatment in surgical wards who had not been operated on and in whom no pronounced changes in the fluid balance had been observed. The fluid balance was checked by determining hemoglobin, hematocrite, plasma bikarbonate and serum protein in blood samples.

METHODS

The blood buffer capacities were measured using a potentiometer (Radiometer Copenhagen, type PHM3,) with a scale readable to the nearest 0.002 pH unit. A freshly prepared acetate buffer, pH 6.50, was used as reference solution.

The measurements were made at 38 C° under anaerobic conditions in an Astrup apparatus on 5 ml samples of undiluted blood which had been collected without their coming into contact with air. The plasma was obtained by centrifuging the blood under a protecting layer of paraffin oil 15 minutes at 3000 rotations per minute.

The buffer capacity values (B.C.) were computed from the van Slyke[2] equation, which when 0.5 ml of 0.1 N hydrochloric acid is added to a 5 ml sample of the solution under test takes the form

$$B.C. = \frac{0.01}{pH_1 - pH_2}$$

where pH_1 is the pH value before, and pH_2 the value after the addition.

In order to evaluate the precision of the buffer capacity values, determinations were made in triplicate on the same sample.

The hemoglobin values were determined by the photometric oxyhemoglobin method. The alkali reserve values were evaluated by the Conway diffusion cell. The serum protein values were obtained with the aid of the biuret reaction.

The glycylglycine was stored in dry infusion bottles and dissolved in isotonic solution immediately before they were used. The buffer capacity of the solution was 0.0379, which corresponds to an extremely high blood buffer capacity.

The buffer capacity of the 6 per cent macrodex infusion solution (dextran 60.0 g; NaCl 9.0 g; Aqua dest. ad 1000.0 ml) was 0.000086, which is more than 400 times as low as that of the 3,74 per cent glycylglycine solution.

RESULTS

The 32 patients who participated as subjects had been selectied as mentioned in the introduction. Twelve of the patients were given only glycylglycine infusions, and eleven patients only macrodex infusions, while nine patients were given whole blood transfusions.

Glycylglycine Infusions. — The twelve patients were given from 18.7 to 56.1 g of glycylglycine (in volumes varying from 500 to 1500 ml) in one infusion. From Table 1 and Fig. 1 it will be noted that in all patients the buffer capacities of both the blood and plasma were higher 6 hours after the infusion than before the infusion. After 24 hours the buffer capacities had decreased to levels close to the original values except in three patients (cases 7, 9 and 12) in whom the values were still definitely higher than

EFFECT OF INFUSED 3.74 PER CENT GLYCYLGLYCINE SOLUTION ON THE BUFFER
CAPACITIES OF BLOOD AND PLASMA

Case No.	Grams Glycyl-Glycine Infused	Buffer Capacity of Blood before and Various Times after the Infusion			Buffer Capacity of Plasma before and Various Times after the Infusion		
		Before	6 hours	24 Hours	Before	6 Hours	24 Hours
1	18.7*	0.0303		0.0308	0.0143		0.0147
2	37.4**	0.0370	0.0388		0.0163	0.0165	
3	»	0.0368	0.0389	0.0368	0.0165	0.0166	0.0165
4	»	0.0323	0.0357		0.0151	0.0161	
5	»	0.0297		0.0300	0.0143		0.0154
6	56.1***	0.0376	0.0400	0.0382	0.0164	0.0175	0.0163
7	»	0.0368	0.0385	0.0400	0.0166	0.0177	
8	»	0.0348	0.0377	0.0353	0.0165	0.0172	0.0169
9	»	0.0345	0.0355	0.0364	0.0164	0.0176	0.0168
10	»	0.0339	0.0370	0.0397	0.0161	0.0178	0.0171
11	»	0.0331	0.0402	0.0355	0.0181	0.0184	0.0169
12	»	0.0321	0.0380	0.0400	0.0155	0.0161	0.0183

* volume 500 ml, ** volume 1000 ml, *** volume 1500 ml.
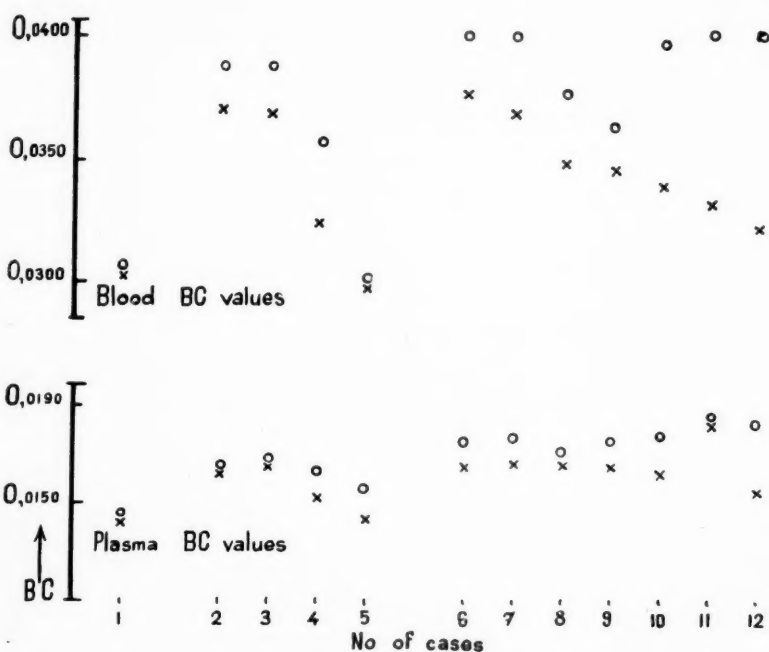
Fig. 1. — Blood and plasma buffer capacities in 12 subjects before and 6—24 hours after glycylglycine infusions. In case 1 18.7 g (in 500 ml), in cases 2—5 37.4 g (in 1000 ml) and in cases 6—12 56.1 g (in 1500 ml) of glycylglycine were infused. x = values of BC before glycylglycine infusions, o = highest values of BC after glycylglycine infusions.

before the infusion. The mean increase in the buffer capacity of
the blood was 9.4 per cent and in that of the plasma 6.3 per cent
for the 6 hour samples.

In this patient group the alkali reserve averaged 48 vol. per
cent CO_2 before the infusion, and 44 vol. per cent CO_2 24 hours
after the infusion.

The mean serum protein values were 6.8 per cent both before
and 24 hours after the infusion.

The mean hemoglobin content was 12.6 g/100 ml before the
infusion, and slightly lower, 12.2 g/100 ml, 24 hours after the
infusion.

The corresponding mean hematocrit values were 42 and 41
per cent.

Macrodex Infusions. — Eleven patients were given 1000 to
1500 ml of macrodex in one infusion (Table 2). Buffer capacity
determinations were made on blood and plasma samples taken
before, 6 hours and 24 hours and in three cases 48 hours after
the infusions.

From Table 2 and Fig. 2 it is seen that the buffer capacities of
the blood and plasma had undergone a decrease in all patients
following infusion. This decrease was greatest 6 hours after the
infusion, but after 24 and 48 hours the values had increased again
close to the levels before the infusion. The greatest mean decrease
in the blood buffer capacity in this group was 7.9 per cent, and
that in the plasma buffer capacity 13.8 per cent. The mean values
have been computed from the lowest values recorded for the
patients.

The mean serum protein content decreased from 7.2 per cent
before, to 6.3 per cent after the infusion. The corresponding mean
hematocrit values were 44 per cent and 42 per cent, and the mean
hemoglobin values 12.7 and 12.1 g/100 ml.

Blood Transfusions. — Volumes of whole blood varying 400
or 800 ml were given by transfusions to 9 patients (Fig. 3). With
one exception the buffer capacity of the blood had increased during
24 hours following the transfusions. The mean increase in buffer
capacity for the nine patients was 6.0 per cent.

The values of the plasma buffer capacity after the infusion
varied for the different patients. A mean decrease in the plasma
buffer capacity of only 3 per cent was found.

TABLE 2

EFFECT OF INFUSED MACRODEX ON THE BUFFER CAPACITIES OF BLOOD AND PLASMA

Case No.	Volume of Mac-rodex Infused	Buffer Capacity of Blood before and Various Times after the Infusion				Buffer Capacity of Plasma before and Various Times after the Infusion			
		Before	6 Hours	24 Hours	48 Hours	Before	6 Hours	24 Hours	48 Hours
1	1000 ml	0.0351	0.0351	0.0348		0.0146	0.0136	0.0156	
2	»	0.0395	0.0375	0.0385		0.0176	0.0157	0.0175	
3	1500 ml	0.0392	0.0385	0.0397		0.0149	0.0141	0.0160	
4	»	0.0392	0.0357	0.0375		0.0174	0.0141	0.0154	
5	»	0.0385	0.0333	0.0357	0.0368	0.0169	0.0141	0.0153	0.0165
6	»	0.0380	0.0345	0.0364		0.0167	0.0139	0.0164	
7	»	0.0385	0.0361	0.0382	0.0385	0.0172	0.0153	0.0159	0.0159
8	»	0.0353	0.0303	0.0333		0.0174	0.0149	0.0167	
9	»	0.0337	0.0306	0.0336		0.0183	0.0143	0.0169	
10	»	0.0296	0.0255	0.0278		0.0153	0.0128	0.0140	
11	»	0.0377			0.0376	0.0169			0.0159
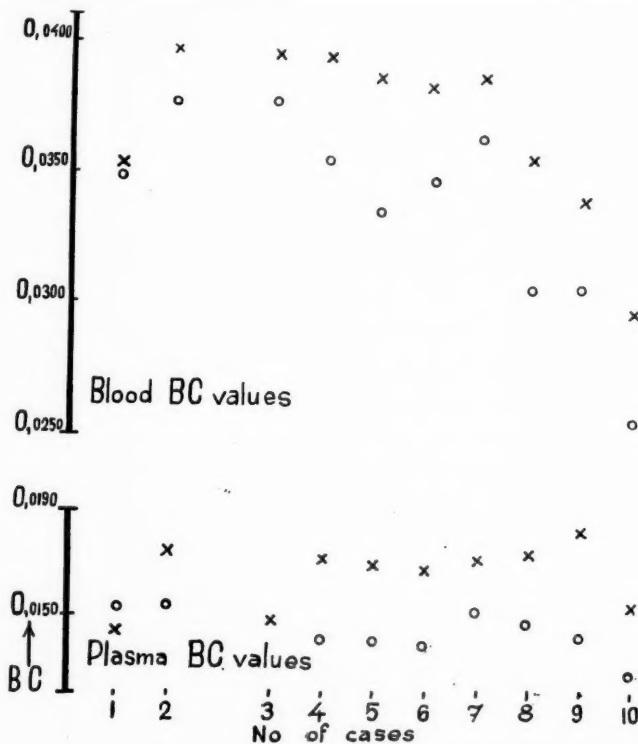
Fig. 2. — Blood and plasma buffer capacities in 10 subjects before and 6—24 hours after macrodex infusions, In cases 1—2 1000 ml of macrodex was infused, in the other cases 1500 ml. x = values of BC before macrodex infusions, o = lowest values of BC after macrodex infusions.
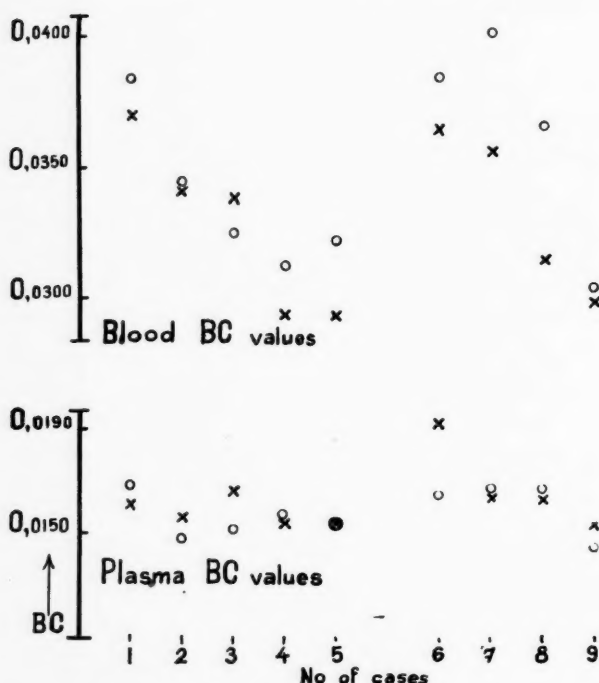
Fig. 3. — Blood and plasma buffer capacities in 9 subjects before and 24
hours after blood transfusions. In cases 1—5 400 ml of whole blood was trans-
fused, in the other cases 800 ml. x = values of BC before blood transfusions,
o = values of BC after blood transfusions.

The following changes were observed in the blood picture in
this group of patients: the mean hemoglobin content rose from
12.5 to 13.5 g/100 ml; the mean serumprotein value from 6.5 to
7.1 per cent; the mean hematocrit value from 36 to 40 per cent.
The mean alkali reserve value underwent no change.

DISCUSSION

The greatest change in the buffer capacities of the blood and
plasma were observed in most cases 6 hours after the infusion of
glycylglycine (Table 1 and Fig. 1). In three cases a higher buffer
capacity was recorded 24 hours after the infusion. Both the mean
alkali reserve and hemoglobin values decreased slightly following
the infusion, while the mean serum protein remained unaltered.

Thus the concentrations of all the factors that are primarily responsible for the maintenance of the buffer capacity of the blood have either decreased or remained unchanged. It is evident that the observed increase in buffer capacity must be ascribed solely to the infused glycylglycine. Since the buffer capacity of the infused 3.74 per cent glycylglycine solution, 0.0379, corresponds to a very high blood buffer capasity, it is redily understood that glycylglycine is able to incriese, although only slightly, the already relatively high buffer capacity from 0.0342 to 0.0374. It may further be assumed that already 6 hours after the infusion a part of the solvent for the glycylglycine has been removed from the circulation and that the active component, glycylglycine, has been retained for the most part in the blood. The effect of glycylglycine appears to persist at least 24 hours after the infusion.

The buffer capacity of the plasma also seems to be increased by glycylglycine, although the increase is relatively slight compared with the increase in the buffer capacity of the blood.

All the patients who were given macrodex infusions were subjects in relatively good health with high blood and plasma buffer capacities. Despite this, one infusion of 1000—1500 ml of macrodex caused in all cases a clear decrease 7.9 per cent on the average in the buffer capacity of the blood. The mean relative decrease in the plasma buffer capacity was even greater, 13.8 per cent. The greatest decrease in buffer capacity was in most cases observed 6 hours after the infusion.

Under ordinary conditions a decrease in the buffer capacity of this order of magnitude is of lesser significance, but if the buffer capacity of the blood of the patient is initially low, liquid infusions during the postoperative period may decrease the buffer capacity to such an extent that alkalemia or acidemia may be imminent (5). This is particularly true if infusions are performed on several days in succession, since, for example, the buffer capacity of macrodex is more than 400 times as small as that of blood or of a 3.74 per cent glycylglycine solution.

In the preparation of infusion solutions, very little attention has been paid to their buffering properties in the range where the pH of the blood varies. There would seem to be reason to add to these solutions glycylglycine or some other substance which exerts a strong buffering action in the pH range of the blood.

In the patients who were given 400 to 800 ml of blood by transfusion, the buffer capacity of the blood increased 6.0 per cent on the average (Fig. 3). During this same period, the buffer capacity of the plasma had not changed, and hence the observed increase in blood buffer capacity must be primarily ascribed to the resulting higher hemoglobin content.

SUMMARY

The variations of the buffer capacities of the blood and plasma have been followed in 32 subjects who were given either 3.74 per cent glycylglycine or macrodex by infusion, or blood transfusions.

The buffer capacities of both the blood and plasma are increased by infusion of glycylglycine. The buffer capacities attain their highest values about 6 hours after the infusion and remain high for at least 24 hours after the infusion.

Blood transfusions also raise the buffer capacity of blood. This is primarily due to increased hemoglobin content.

The buffer capacity of blood decreased in all subjects who were given macrodex by infusion. An even grater decrease was noted in the buffer capacity of the plasma.

Since the buffer capacities of infusion solutions have not received adequate attention, it is purposed that compounds similar to glycylglycine with a strong buffering action in the pH range in question be added to these solutions.

REFERENCES

1. KILPI, S.: Acta chem. Scandinav. 1952:6:475.
2. VAN SLYKE, D. D.: J. Biol. Chem. 1922:52:522.
3. VIIKARI, S. J.: Acta chir. Scandinav. 1954:107:525.
4. VIIKARI, S. J., and KLOSSNER, O.: Acta chir. Scandinav. 1955:109:222.
5. VIIKARI, S. J., and SALENIUS, P.: Ann. med. exp. et biol. Fenniae 1957:35:16.

METHODS OF DETERMINING THE BUFFER CAPACITY OF BLOOD

by

S. J. VIIKARI and O. KLOSSNER

Together with the pre- and postoperative care of surgically treated patients greater regard has lately been paid to the *pH* and the buffer capacity of the blood.(2)

The living organism tends to maintain a constant degree of acidity which is essential condition for undisturbed vital functions. As is known, for instance, deficient burning in the organism results in acid burning products. That the reaction of the blood might remain unchanged, certain regulative mechanisms are needed. One of these is the buffer system of the blood. Hemoglobin is by far the most important component of this buffer system corresponding to 75% of the total buffer capacity.

METHODS

The determination of the buffer capacity is a relatively easy procedure. By means of a potentiometer the *pH* is determined in given amount of blood. A fixed quantity of acid is added after which the *pH* is determined anew. The less the two *pH* values differ the greater is the buffer capacity and vice versa.

It is well known that changes take place in the *pH* of the blood if the blood temperature is not constant (1, 3). For accurarte *pH* measurements, therefore, it is necessary to use a thermostat (37°C) and to draw the blood sample in such a manner that contact with air is not possible.

The aim of this paper has been to study the effect of air and temperature on the buffer capacity values. The writers have carried out parallel determinations of the buffer capacity of the blood by three different methods.

1) In one of these methods the blood *pH* was measured under anaerobic conditions at the temperature of 37°C. An amount of 5 ml heparinized anaerobically drawn blood was placed under paraffin. The *pH* was measured with a *pH* meter accurate to 0.002 *pH*-units. The temperature was kept constant by means of a water thermostat constructed by Astrup. After the addition of 0.5 ml of 0.1-n hydrochloric acid — still anaerobically — the *pH* was determined anew and the buffer capacity was calculated from the difference in the *pH* values (2).

$$\text{Buffer capacity} = \frac{0.01}{pH_1 - pH_2}$$

2) By applying the above mentioned anaerobic technique but at room temperature.

3) The blood sample was exposed to the influence of air at room temperature.

Apart from the above mentioned differences the three determinations were carried out in exactly the same way.

MATERIAL AND RESULTS

For comparison of the three methods of determination parallel determinations of the buffer capacity of the blood were made from 40 blood samples. The samples were taken from surgical patients either pre- or postoperatively. A sufficiently large variation in the buffer capacity values has thus been obtained.

The values obtained by the three methods are shown in table 1.

As the table indicates the three analysis methods gave nearly identical buffer capacity values. The slight differences observed are probably due to inaccurate measuring. We know that even the slightest error in the measurement of the added acid will cause a considerable change in the corresponding buffer capacity value. For this reason every effort was made in these experiments to measure the added acid as accurately as possible by means of a micropipette.

TABLE 1

THE BUFFER CAPACITY VALUES OBTAINED BY THE THREE DIFFERENT METHODS

Case	I	II	III	Case	I	II	III
1	0.025	0.026	0.025	21	0.026	0.026	0.026
2	0.033	0.033	0.033	22	0.026	0.026	0.024
3	0.034	0.034	0.032	23	0.031	0.031	0.030
4	0.031	0.033	0.032	24	0.030	0.030	0.029
5	0.030	0.031	0.030	25	0.029	0.029	0.028
6	0.034	0.036	0.034	26	0.033	0.034	0.034
7	0.036	0.037	0.037	27	0.029	0.029	0.031
8	0.033	0 032	0.033	28	0.033	0.033	0.034
9	0.034	0.033	0.033	29	0.030	0.030	0.033
10	0.037	0.036	0.037	30	0.030	0.030	0.031
11	0.029	0.029	0.029	31	0.030	0.030	0.030
12	0.028	0.028	0.028	32	0.028	0.028	0.029
13	0.033	0.033	0.034	33	0.027	0.029	0.028
14	0.033	0.033	0.032	34	0.029	0.029	0.028
15	0.028	0.028	0.029	35	0.037	0.037	0.036
16	0.028	0.028	0.029	36	0.031	0.031	0.027
17	0.030	0.031	0.030	37	0.036	0.036	0.034
18	0.029	0.031	0.031	38	0.033	0.033	0.033
19	0.032	0.033	0.032	39	0.031	0.031	0.029
20	0.032	0.031	0.031	40	0.034	0.034	0.034

The buffer capacity determinations were carried out by three different methods:

I under anaerobic conditions, at the temperature of 37°C
II under anaerobic conditions, at room temperature
III under aerobic conditions, at room temperature

No tendency whatever to higher or lower values was noticed in any of the methods. The reason why the effect of air and temperature can be ignored is probably due to the fact that the buffer capacity values are calculated from the difference of two *pH* values both of which are influenced by the same factors.

SUMMARY

The authors made parallel determinations of the buffer capacity of the blood in surgical patients using three techniques.

One of the methods consisted in the determination of the blood buffer capacity under anaerobic conditions from blood samples maintained at a constant temperature of 37°C. In the second

method the measurement was carried out under anaerobic conditions but at room temperature. In the third method the buffer capacity was determined under aerobic conditions at room temperature.

The three methods gave similar results. The aerobic technique has greater facility and therefore is better adapted to clinical work.

REFERENCES

1. ROSENTHAL, T. B.: J. Biol. Chem. 1948:*173*:25.
2. VIIKARI, S. J.: Acta chir. Scand. 1954:*107*:525.
3. WILSON, R. H.: J. Lab. & Clin. Med. 1951:*37*:129.

UROPEPSIN EXCRETION IN PARTIALLY GASTRECTOMIZED PEPTIC ULCER PATIENTS WITH POSTCIBAL SYMPTOMS

by

S. J. VIIKARI and O. CASTRÉN

The proteolytic enzyme urinary pepsinogen or uropepsin which is excreted into the urine from the kidneys has been known for a long time. This enzyme, which is active in the acid *pH* range, seems to be identical with gastric pepsinogen secreted by glands in the gastric mucus membrane, part of which enters the blood stream and is transported to the kidneys.

The normal excretion of uropepsinis considered to be 20—40 units/hour. When total gastrectomy has been performed, no uropepsin excretion is detected in the urine (2, 8). Neither have persons suffering from achlorhydria and pernicious anemia been found to excrete this enzyme in the urine.

It has been stated that the uropepsin excretion parallels the gastric acid secretory activity (5). This has led to the opinion that the determination of urinary uropepsin level is of diagnostic value (4, 5, 8, 9), for it has been established that the rate of uropepsin excretion is high in peptic ulcer patients.

Cubberly and his co-workers (3) have found that partial gastrectomy lowers the uropepsin excretion of peptic ulcer patients. A number of other authors, among them Asher (1), have however stated that partial gastrectomy does not affect the excretion.

Spiro and co-workers (10) established a clearly increased uropepsin excretion following administration of corticotropin. Asher (1)

has drawn attention to the increased uropepsin excretion under conditions of stress; the stress need not be emotional, but may be physical or hormone-induced (6).

METHOD

The method used in this investigation for the determination of urinary uropepsin is that described by West and his co-workers (11). The determinations were made on 24-hour urine samples obtained from subjects who were given careful instructions for the collection of the samples. Toluene was added to each of the samples to prevent deterioration, and the uropepsin levels were determined within 48 hours after the samples had been collected.

Reagents: Acetate buffer, *pH* 4.90 (NaOH 4.2 g, glacial acetic acid 9.2 ml, distilled water to make 100 ml).

2N hydrochloric acid.

0.2 per cent aqueous methyl orange solution.

Milk-buffer mixture. One part of freshly homogenized milk prepared from 8 g of milk powder and 200 ml of distilled water and one part of acetate buffer were mixed. The activation of uropepsin was effected by adding 0.05 ml of methyl orange solution and 0.10 ml of 2N HCl to 2 ml of the urine; the resulting solution should be acid to methyl orange, i.e. *pH* 3 or less. The above volume of hydrochloric acid was found sufficient in all cases. The acidified urine was warmed one hour in a 37°C water bath. All reagents and test tubes were also warmed in the same bath. One-tenth-ml of activated urine was measured into a test tube, and 0.90 ml of distilled water and 1.0 ml of acetate buffer were added, followed by 0.50 ml of the milk-buffer mixture. After mixing the contents, the tube was transferred to the thermostat. The end points of the precipitation of casein particles was determined by viewing the tube against a lamp. The time required for complete precipitation can be measured with an accuracy of a few seconds.

The results are expressed as units of uropepsin excretion per hour:

units/hour $= \dfrac{10\,V}{hvs}$; V is the total volume of urine collected in h hours,

v ml the volume of activated urine used in the test, and s the number of seconds required to reach the end point.

RESULTS

The purpose of the study was to determine whether the uropepsin excretion rises or falls in peptic ulcer patients after partial gastrectomy. Particular attention has been paid to partially gastrectomized patients with moderate or severe postcibal symptoms (Table 1). For comparison, the excretion of uropepsin was

TABLE 1

UROPEPSIN LEVELS IN GASTRODUODENAL ULCER PATIENTS WITH POSTCIBAL
SYMPTOMS AFTER PARTIAL GASTRECTOMY

Case	Sex	Nature of Ulcer	Grade of Postcibal Symptoms	Neurolability	Extent of Resection	Time from Resection (Years)	Units of Uropepsin per Hour
1	Male	Gastric	Pc 2	+	2/3 resection	3	105.5
2	»	Duodenal	Pc 1	+	»	6	99.6
3	Female	»	»	+	»	3	95.8
4	Male	Gastric	»	—	»	3	83.6
5	»	»	»	—	»	5	80.6
6	»	Duodenal	Pc 2	++	»	3	80.3
7	Female	»	»	+	»	8	73.6
8	Male	»	Pc 1	—	»	8	69.3
9	»	Gastric	»	—	»	9	66.7
10	Female	Duodenal	»	+	»	5	64.1
11	»	»	»	+	»	2	59.4
12	Male	»	Pc 2	+	»	7	57.8
13	»	Gastric	»	+	»	3	56.6
14	Female	»	Pc 1	+	»	5	54.0
15	Male	»	»	+	»	5	53.9

Mean 73.4 ± 4.4

Case 12 had developed recurrent ulcer and vagotomy had been performed 3 years previously.

TABLE 2

UROPEPSIN LEVELS IN GASTRODUODENAL ULCER PATIENTS WITHOUT SYMPTOMS
AFTER PARTIAL GASTRECTOMY AND IN HEALTHY CONTROLS

	No. of Cases	Average Amounts of Uropepsin Excreted (Units/ Hour)
Partial gastrectomized patients without symptoms	9	53.7
Subjects with no gastroduodenal symptoms	4	50.4
	13	Mean 52.7 ± 4.4

followed in partially gastrectomized patients with no postoperative symptoms and in subjects with no symptoms of gastrointestinal tract origin (Table 2).

In 15 patients with postcibal symptoms, the uropepsin excretion varied from 105.5 units/hour to 53.9 units/hour. The average

excretion was 73.4 ± 4.4 units/hour. The individual values are given in Table 1.

In nine peptic ulcer patients who had no symptoms after partial gastrectomy performed from 2 to 4 years previously the highest uropepsin excretion was 91.7 units/hour and the lowest 36.6 units/hour. The mean value was 53.7 units/hour.

In the four persons with no gastrointestinal symptoms the highest value was 63.4 units/hour and the lowest 30.7 units/hour. The mean was 50.4 units/hour.

DISCUSSION

The highest »normal» urinary excretion of uropepsin is considered to be 40 units/hour, but in studies of the normal excretion marked individual variations have been observed. For example, Janowitz and co-workers (8) found for a control series of 44 subjects a mean excretion of 47 units/hour, with a range from 0 to 136 units/hour.

For the control cases of the present study, the mean uropepsin excretion was slightly higher than previously reported mean values for the uropepsin excretion (Table 2).

The values found for the nine partially gastrectomized peptic ulcer patients who had no postoperative symptoms are in accord with the opinions of Gray and co-workers (7) and Asher (1) that partial gastrectomy does not alter the uropepsin excretion. The low values reported by Cubberly and co-workers (3) for such patients thus appear to be erroneous.

Among the patients with postcibal symptoms, the mean uropepsin excretion 73.4 ± 4.4 units/hour was slightly, but definitely, higher than normal. Comparison of the groups cited in Table 1 and 2 showed also that the differences were statistically significant. Asher (1) and Gray and co-workers (6) have found conditions of stress to be associated with high levels of uropepsin excretion. Patients with postcibal symptoms are generally characterized as being vaso- or neurolabile (12), and they frequently suffer from »chronic stress». Emotional instability was clearly evident in some of the patients examined in this study. The labile psychic conditions and jejunal irritation after meals in the patients with postcibal symptoms may be factors that maintain chronic stress.

Whereas Cubberly and co-workers (3) have stated th at anti-cholinergic drugs lower the excretion of uropepsin, Asher (1) has expressed the opinion that these drugs have no such effect. Acc ording to Gray and co-workers (7) vagotomy does not influence t he uro-pepsin excretion. Among the authors' cases there was one (c ase 12) on whom vagotomy for recurrent ulcer had been performed three years previously; his uropepsin excretion was 57.8 units/hour.

SUMMARY

The mean uropepsin excretion in 15 partially gastrectomized peptic ulcer patients with postcibal symptoms was found to be 73.4 ± 4.4 units per hour. This figure is higher than that found for four subjects without any symptoms in the gastrointestinal tract. This figure is also higher than that found for nine partialy gastrectomized peptic ulcer patients without any postoperative symptoms. The authors conclude that labile psychic conditions and jejunal irritation after meals maintain chronic stress in patients with postcibal symptoms which results in an increased urinary excretion of uropepsin.

REFERENCES

1. ASHER, L.: Gastroenterology 1955:*29:*136.
2. BUCHER, G. R., and IVY, A. C.: Am. J. Physiol. 1947:*150:*415.
3. CUBBERLY, D. A., DAGRADI, A., CAINE, H. O., and STEMPIEN, S. J.: Gastroenterology 1955:*28:*80.
4. FARNSWORTH, E. B., SPEER, E., and ALT, H. L.: J. Lab. & Clin. Med. 1946:*31:*1025.
5. GOTTLIEB, E.: Skand. Arch. Physiol. 1924:*46:*1.
6. GRAY, S., BENSON, J. A., REIFENSTEIN, R. W., and SPIRO, H. M.: J. A. M. A. 1951:*147:*1529.
7. GRAY, S. J., RAMSEY, C. G., and REIFENSTEIN, R.: New England J. Med. 1954:*251:*835.
8. JANOWITZ, H. D., LEVY, M. H., and HOLLANDER, F.: Am. J. Med. Sc. 1950:*220:*679.
9. PODORE, C. J., BROH-KAHN, R. H., and MIRSKY, I. A.: J. Clin. Invest. 1948:*27:*834.
10. SPIRO, H. M., REIFENSTEIN, R. W., and GRAY, S. J.: J. Lab. & Clin. Med. 1950:*35:*899.
11. WEST, P. M., ELLIS, F. W., and SCOTT, B. L.: J. Lab. & Clin. Med. 1952:*39:*159.
12. VIIKARI, S. J., and KLOSSNER, O.: Acta Chir. Scand. In Press.

SENSITIVITY OF THE GENUS PROTEUS TO SULPHONAMIDES

by

ILMARI PALVA and W. J. KAIPAINEN

Sensitivity determinations continue to reveal the remarkable chemoresistance of the genus Proteus (2, 5, 6, 9). Some 50 per cent of the Proteus strains submitted laboratories for sensitivity determination come from urinary tract infections (2, 6, 9). Mostly, they come from complicated urinary tract infections (10). Sulphonamides are the general therapy used (1, 4, 13), although fairly effective drugs, recommended for use in Proteus infections (5, 6, 7), have been found among the broad-spectrum antibiotics discovered later.

The sensitivity of bacteria to sulphonamides has been determined almost exclusively by the disc plate method (5, 9); the culture medium must not contain peptone (8, 14) which inhibits the action of sulphonamides. The effectiveness of sulphonamides is also known to be reduced by urine. Thus urine cannot be saturated in vitro by sulfathiazole concentrations high enough to inhibit the growth of microorganisms (12). This notwithstanding, sulphonamides have been used successfully against urinary tract infections. The most important factor in vivo is considered to be the least possible acetylization of the sulphonamides (13). Sensitivity determinations on plate provide only a relative idea of the sensitivities of microorganisms although correctly interpreted, they have proved helpful (3).

The object of the present investigation was to study the sulphon-amide sensitivities of Proteus strains isolated from urinary tract infections and to find whether a correlation exists between the Proteus type and sensitivity to sulphonamides.

The Proteus strains were collected from urine samples, taken in sterile conditions, submitted to the Department of Serology and Bacteriology of Helsinki University, including samples from both acute and chronic urinary tract infections.

PROTEUS TYPING

Proteus typing was based on the following formula (15):

	Saccha-rose	Malt-ose	Sali-cin	Man-nit	Indole	H_2S	Gela-tine	Urea
Proteus mirabilis	×	—	×	—	—	+	+	+
Proteus vulgaris	+	+	+	—	+	+	+	+
Proteus rettgeri	×	—	+	+	+	—	—	+
Proteus morganii	—	—	—	—	+	×	—	+

+ positive, — negative, × variable.

Previously, the commonest type found in Proteus infection used to be the Proteus vulgaris, as the name indicates. The present classification method, however, shows Proteus mirabilis to be the commonest (5, 6), due to the fact that Proteus mirabilis was classified as indole-negative Proteus vulgaris. We arrived at the same result in the distribution of the present material (Table 1).

TABLE 1
DISTRIBUTION BY TYPES OF PROTEUS STRAINS ISOLATED FROM URINARY TRACT INFECTIONS

Proteus mirabilis	50	62.5	per cent
Proteus vulgaris	16	20.0	» »
Proteus rettgeri	8	10.0	» »
Proteus morganii	6	7.5	» »
Total	80	100.0	

METHOD OF SENSITIVITY DETERMINATION

We preferred to make the sensitivity determinations in liquid culture medium in tubes, in order to be able to determine on each occasion the minimum growth-inhibiting concentration, which gives values that are better comparable. Two mediums were employed

parallelly, viz. broth and a modified minimum medium containing a small amount of broth.

Composition of broth			Minimum medium, modified		
NaCl	5.0		K_2HPO_4	3.5	
Liebig's meat extract	3.0		KH_2PO_4	1.5	
Peptone	10.0		$(NH_4)_2SO_4$	0.5	
Aq.dest.	ad 1000.0		$MgSO_4.2H_2O$	0.05	
pH 7.0			NaCitr.	0.25	
			Vitamin B_{12}	0.000005	
			Broth	0.5 ml	
			Glucose	5.0	
			Aq. dest.	ad 500.0	
			pH 7.0		

The glucose was added to the minimum medium after autoclaving. The following sulphonamides were used:

Lucosil	(6-sulfanilamido-2,4-dimethylpyridin)
Elkosin	(2-sulfanilamido-5-methyl-1,3,4-thiodiazol)
Gantrisin	(3,4-dimethyl-5-sulfanilamidoisoxazol)

A basic solution, 200 mg/ml of sterile saline solution, was made of each sulphonamide from sterile commercial ampoules. The solution was stored frozen. Fresh solutions were prepared weekly. A serial two-fold dilution of each sulphonamide was made into inoculated culture medium. The amount of inoculum was a loopful per 15 cc of medium. It was not considered possible to use higher concentrations than 1000 mg per 100 ml because of the problem of solubility. The incubation period was 20 hours at 37°C. Readings were taken by the naked eye. The sensitivity of each strain tested was taken to equal the minimum sulphonamide concentration inhibiting visible growth.

RESULTS

It was to be expected that no great differences in inhibitory concentration would be obtained between different strains in experiments with broth, due to the large amount of peptone inhibiting the activity of the sulphonamidies. However, as some differences were noted the sensitivity determinations in broth were continued regularly. Fig. 1 shows a comparison of the sensitivity to Lucosil of all the Proteus strains tested, in broth and in the minimum
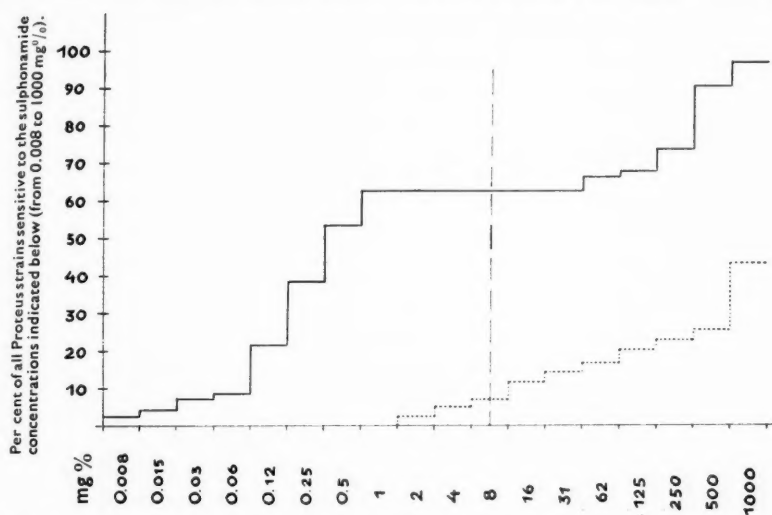
Fig. 1. — Comparison of the sensitivities of 80 Proteus strains to Lucosil by twofold serial method using (a) broth ············· or (b) modified minimum medium ——— as culture medium. The vertical broken line indicates the average blood level after normal dosage.
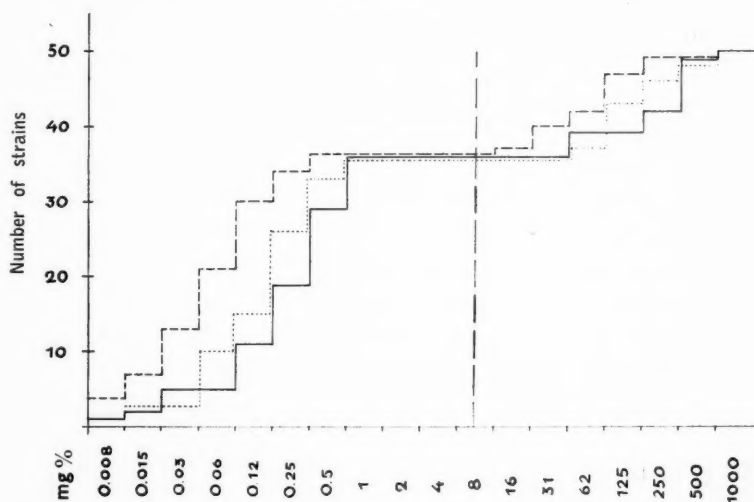
Fig. 2. — Numerical distribution of 50 Proteus mirabilis strains by their sensitivity to the concentrations of Lucosil, Elkosin and Gantrisin listed above (from 0.008 to 1000 mg%). The vertical broken line indicates the average blood level of each of the drugs.

medium. It is distinctly observable that the inhibitory concentrations in broth are considerably higher, and that 1000 mg% of Lucosil was capable of inhibiting the growth of only 43 per cent of the strains in broth. In addition, 2 mg% was found to be the minimum concentration giving growth inhibition. From this amount upwards the number of strains inhibited increased evenly with increasing concentration. In the minimum medium very small concentrations even were capable of inhibiting the growth of some strains. On reaching concentrations of 0.12—1.0 mg% the number of strains inhibited increases rapidly, after which there is a broad plateau constituting a very marked, extensive demarcation zone for the more resistant strains. With this difference in the results of the material in the two culture mediums it is obvious that experiments in broth alone are not capable of giving nearly so variable a picture of the distribution of the sensitivities of the different Proteus types as experiments in minimum medium. As the sensitivity results for Elkosin and Gantrisin using broth as culture medium were similar to those for Lucosil, we have not considered it of interest to draw figures for them.

Fig. 2 shows the distribution of the sensitivities to Elkosin, Lucosil and Gantrisin of 50 Proteus mirabilis strains. In the same way as in Fig. 1, there is a demarcation zone from the 1 mg% level upwards. The same extensive dividing zone, free of variations in sensitivity, is obtained for all the sulphonamides. There are plenty of strains extremely sensitive to all three sulphonamides. With increasing concentration the number of strains inhibited also increases rapidly. Some differences are observable between the various sulphonamides. Elkosin ·and Lucosil are very similar, whereas more strains are sensitive to Gantrisin. On reaching 1 mg%, however, the number of sensitive strains is exactly the same for all three drugs. Only after crossing the broad demarcation plateau are there strains that are sensitive to 16—31 mg%, from which level onwards the number of strains inhibited increases with increasing concentrations.

Fig. 3 illustrates the conditions for Proteus vulgaris. In the sensitive area, more strains are sensitive to Gantrisin than to the others; the majority, however, are sensitive to 1 mg% of each sulphonamide. There were fewer strains than in the previous group, but in our opinion the distribution shows clearly that Proteus
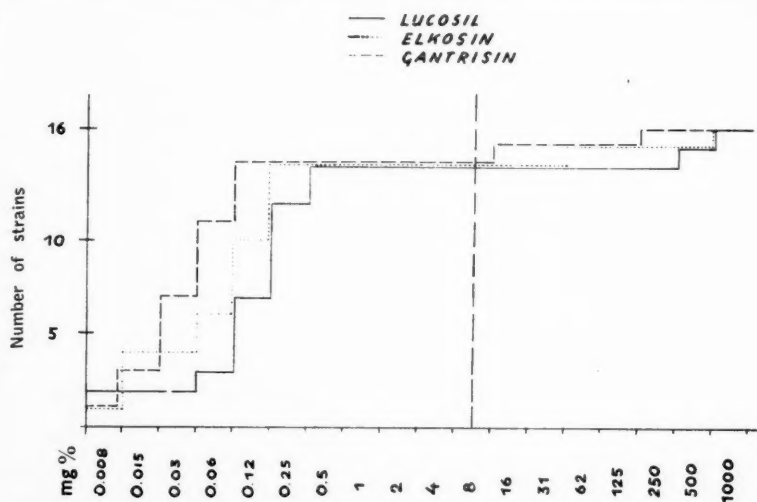
Fig. 3. — Numerical distribution of 16 Proteus vulgaris strains by their sensitivity to the concentrations of Lucosil, Elkosin and Gantrisin listed above (from 0.008 to 1000 mg%). The vertical broken line indicates the average blood level of each of the drugs.
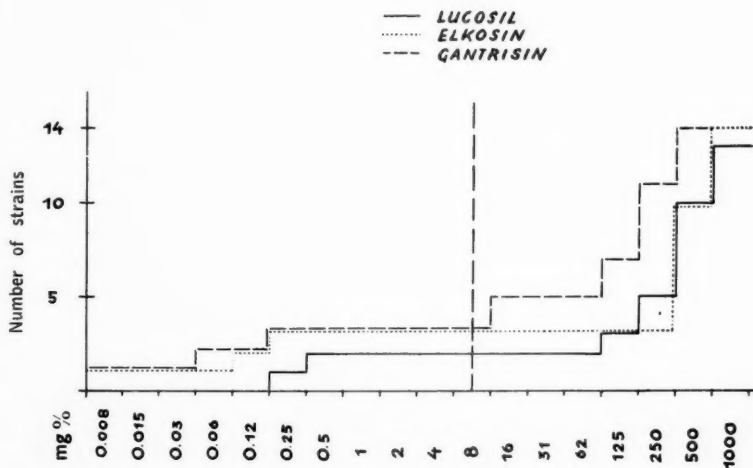
Fig. 4. — Numerical distribution of 14 Proteus rettgeri and P. morganii strains by their sensitivity to the concentrations of Lucosil, Elkosin and Gantrisin listed above (from 0.008 to 1000 mg%). The vertical broken line indicates the average blood level of each of the drugs.

vulgaris is on the whole sensitive more often than resistant to Lucosil, Elkosin and Gantrisin.

The Proteus rettgeri and Proteus morganii strains were dealt with together, as they are few and seemed to behave similarly. Fig. 4 shows that an extensive demarcation zone was observable for them too. However, the bulk of the Proteus rettgeri and Proteus morganii strains were in the resistant area. Gantrisin gave a slightly better result in this group also.

CONCLUSIONS AND SUMMARY

Of eighty Proteus strains isolated from urinary tract infections, 62.5 per cent were Proteus mirabilis, 20 per cent Proteus vulgaris, 10 per cent Proteus rettgeri, and 7.5 per cent Proteus morganii.

Their sensitivity to Elkosin, Lucosil and Gantrisin was tested. No appreciable differences in the sensitivity of the strains of the different types were obtained in broth with a high peptone content. In a minimum medium, with a small amount of peptone, definite differences in sensitivity were observable between the different types. 72 per cent of the mirabilis strains were sensitive to 1 mg% of Elkosin, Lucosil and Gantrisin. A rise in the sulphonamide concentration from 1 mg% to 16 mg% produced no increase in the number of strains. From the 16 mg% level onwards the number of strains inhibited increases with increasing concentrations. Gantrisin seemed slightly more effective than Lucosil and Elkosin both in the sensitive and the resistant areas.

87.5 per cent of the Proteus vulgaris strains were sensitive to 1 mg% of Elkosin, Lucosil and Gantrisin. Strengthening the concentration from 1 mg% to 16 mg% had no effect on the number of sensitive strains.

Against Proteus morganii and Proteus rettgeri all the sulphonamides were ineffective in almost the same degree, although some sensitive strains were found among them.

REFERENCES

1. Buchtel, H. A., and Cook, E. N.: Proc. Staff. Meet. Mayo Clin. 1937: 12:444.
2. Frank, P. F., Wilcox, C., and Finland, M.: J. Lab. & Clin. Med. 1950:35:205.
3. Kaipainen, W. J., and Parmala, M. E.: Ann. Med. Int. Fenniae 1953:42:266.
4. Narins, L.: J. Urol. 1948:59:92.
5. Perch, B.: Acta Path. & Microb. Scand. 1954:35:278.
6. Potee, K. G., Wright, S. S., and Finland, M.: J. Lab. & Clin. Med. 1954:44:463.
7. Rhoads, P. S., Billings, C. E., and O'Conor, V. J.: J.A.M.A. 1952: 148:165.
8. Sesler, C. L., and Schmidt, L. H.: J. Pharmacol. 1943:79:117.
9. Story, P.: J. Path. & Bact. 1954:68:55.
10. Streng, H. O.: Acta Soc. Med. Fenn. Duodecim Ser. A. Tom. XIX, 1936.
11. Thjøtta, Th.: Laerebok i Bakteriologi, Bind II. — Fabritius & Sønner Forlag, Oslo, 1942:261.
12. Tuovinen, P. I.: Ann. Med. Exp. et Biol. Fenniae 1949:27:128.
13. Walter, A. M., and Heilmeyer, L.: Antibiotika-Fibel, — G. Thieme Verlag, Stuttgart 1954:22.
14. While, H. J., Lictfield, J. T., Jr., and Marshall, E. K., Jr.: J. Pharmacol. 1941:73:104.
15. Winkle, S.: Mikrobiologische u. Serologische Diagnostik, Gustav Fischer Verlag, Stuttgart 1955:63.

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SENSITIVITY OF THE GENUS PROTEUS TO ANTIBIOTICS

by

ILMARI PALVA and W. J. KAIPAINEN

In an earlier work (2) the authors found that the sensitivities to Lucosil, Elkosin and Gantrisin of Proteus strains isolated from urinary tract infections depend considerably on their type. 72 per cent of the Proteus mirabilis and 87.5 per cent of the Proteus vulgaris strains were classified as sensitive in vitro to the above sulphonamides. On the other hand, the Proteus rettgeri and Proteus morganii strains were in the main resistant, but they only accounted for 17.5 per cent of the total material. In the distribution of Proteus strains into sensitive or resistant strains it was striking that the distinction was very clear and there was no intermediate group between sensitive and resistant. In other words, no strains could be called moderately sensitive or moderately resistant. Although in vitro sensitivity determinations are greatly dependent on the method by which they are made and they cannot nearly always correspond to the conditions in vivo, they may when correctly interpreted be of assistance to the clinician. Naturally, it should be borne in mind that especially in chronic urinary tract infections a successful conquest of the infection depends primarily on whether the pathologic conditions can be put right otherwise (4).

This is a report of the sensitivities of 80 Proteus strains to penicillin, dihydrostreptomycin, chloramphenicol, tetracycline, aureomycin and terramycin. Attention has also been paid to whether or not the sensitivities were dependent on the Proteus type.

MATERIAL AND TECHNIQUE OF SENSITIVITY DETERMINATION

The Proteus strains were isolated from urinary tract infections, both acute and chronic. Typed according to Winkle (5), the distribution of these 80 Proteus strains was: Proteus mirabilis 62.5 per cent, Proteus vulgaris 20 per cent, Proteus rettgeri 10 per cent, and Proteus morganii 7.5 per cent. The culture medium used in the sensitivity determinations was the following modified minimum medium:

$$
\begin{array}{ll}
K_2HPO_4 & 3.5 \\
KH_2PO_4 & 1.5 \\
(NH_4)_2SO_4 & 0.5 \\
MgSO_4 \cdot 2H_2O & 0.05 \\
NaCitr & 0.25 \\
Vitamin\ B_{12} & 0.000005 \\
Broth & 0.5\ ml \\
Aq.dest. & ad\ 500.0\ (pH\ 7.0)
\end{array}
$$

5.0 g of sterile glucose was added after autoclaving. 15 ml of the test medium was inoculated with a loopful of an overnight broth culture of the organism to be tested and a twofold serial dilution of the antibiotic was prepared in tubes with the inoculated medium. The incubation period was 20 hours, temperature $+37°C$. The minimum concentration of the antibiotic preventing visible growth was taken as the sensitivity of the strain tested.

RESULTS

Figs. 1—3 show the distribution of the sensitivities of the different Proteus types to chloramphenicol, dihydrostreptomycin and penicillin. Due to the paucity of the strains, the sensitivities of the Proteus rettgeri and Proteus morganii strains are shown in one diagram (Fig. 3). A comparison of these three diagrams reveals a considerable degree of similarity between the results, i.e. the distribution of sensitivities does not depend on the type of Proteus but on the antibiotic used. In each of Figs. 1—3 it is seen that chloramphenicol was definitely more effective than dihydro-streptomycin, and the latter equally superior to penicillin. Although the penicillin units in Figs. 1—3 may not be fully comparable with the dihydrostreptomycin and chloramphenicol values ($\mu g/ml$), the weakness of penicillin is nevertheless striking.
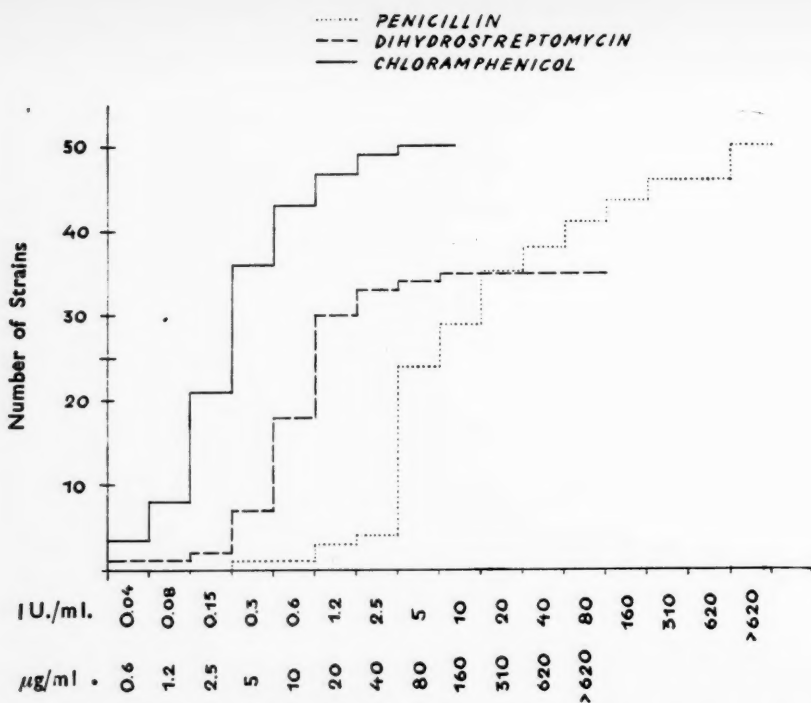
PENICILLIN
DIHYDROSTREPTOMYCIN
CHLORAMPHENICOL

Fig. 1. — The numerical distribution of 50 Proteus mirabilis strains by their sensitivity to the above concentrations of penicillin (I.U./ml), dihydrostreptomycin and chloramphenicol (μg/ml).

PENICILLIN
DIHYDROSTREPTLCMYCIN
CHLORAMPHENICO
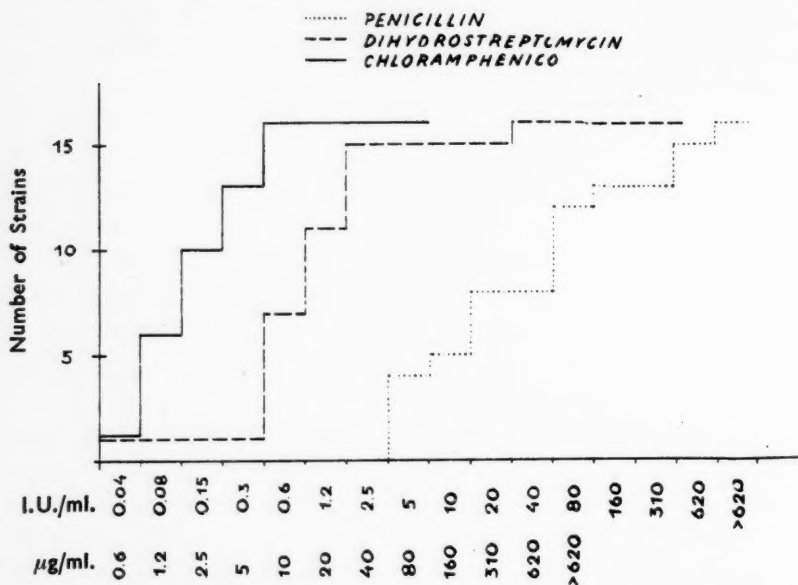
Fig. 2. — The numerical distribution of 16 Proteus vulgaris strains by their sensitivity to the above concentrations of penicillin (I.U./ml), dihydrostreptomycin and chloramphenicol (μg/ml).
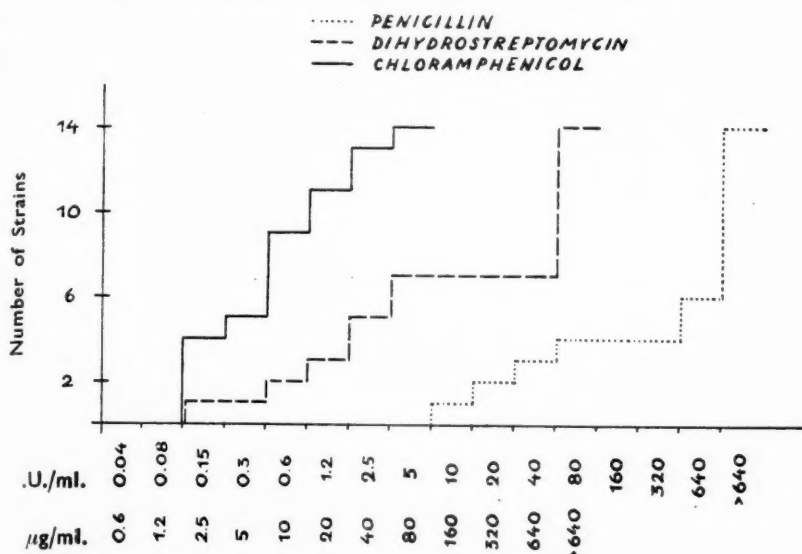
Fig. 3. — The numerical distribution of 14 Proteus rettgeri and Proteus morganii strains by their sensitivity to the above concentrations of penicillin (I.U./ml.), dihydrostreptomycin and chloramphenicol (μg/ml).

Figs. 4—6 show the sensitivities of the Proteus strains to the tetracycline group. The sensitivities to aureomycin, terramycin and tetracycline show a similar distribution, irrespective of Proteus type, although the Proteus rettgeri and Proteus morganii strains are slightly more resistant than the Proteus mirabilis and Proteus vulgaris strains. Minor differences are observable between the antibiotics. Tetracycline is somewhat more effective than aureomycin and terramycin. Moreover, the sensitivities of the Proteus strains to the tetracycline group seem to be of the same order as to dihydrostreptomycin.

TABLE 1

NUMBER AND PERCENTAGE OF PROTEUS STRAINS (OF A TOTAL OF 80) WHICH WERE SENSITIVE TO DIFFERENT ANTIBIOTICS

					Number	Percentage
Sensitive to 0.6	I.U./ml.		Penicillin		1	1.3
»	»	10	μg/ml	Dihydrostreptomycin	27	33.8
»	»	10	»	Chloramphenicol	68	85.0
»	»	5	»	Aureomycin	19	23.7
»	»	5	»	Terramycin	17	21.2
»	»	5	»	Tetracycline	29	36.2
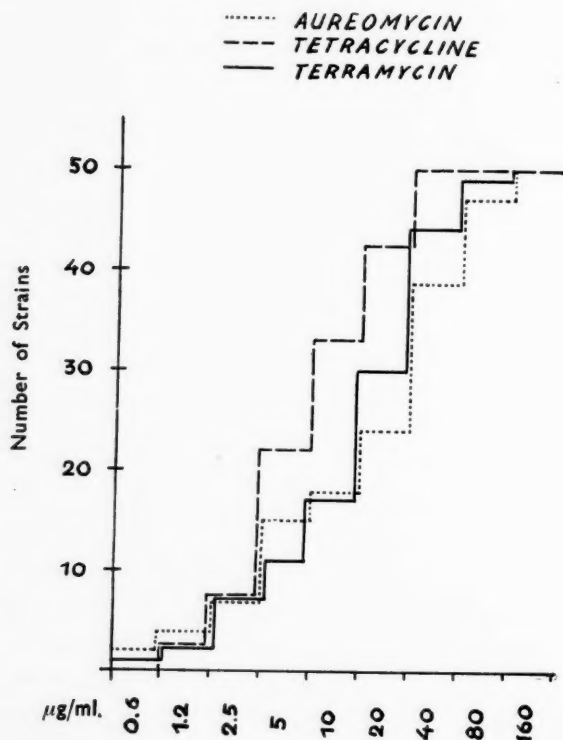
Fig. 4. — The numerical distribution of 50 Proteus mirabilis strains by their sensitivity to the above concentrations of aureomycin, tetracycline and terramycin (μg/ml).

A comparison of these results with the mean antibiotic concentrations obtainable in the blood by normal dosage (Table 1) shows that 85 per cent of all Proteus strains, irrespective of type, are sensitive to 10 μg/ml of chloramphenicol. Similarly, 33.8 per cent of the strains are sensitive to 10 μg/ml of dihydrostreptomycin. Assuming an average blood level of 5 μg/ml for tetracycline, aureomycin and terramycin, 36 per cent of the strains are sensitive to tetracycline, 23.7 per cent to aureomycin, and 21 per cent to terramycin. One strain only can be considered sensitive to penicillin.

Table 2, which gives the sensitivities of the Proteus strains compared with the serum concentrations after maximal antibiotic dosage, shows that only 65 per cent of the strains were resistant to 5 I.U./ml of penicillin. Since it is possible specifically with penicillin
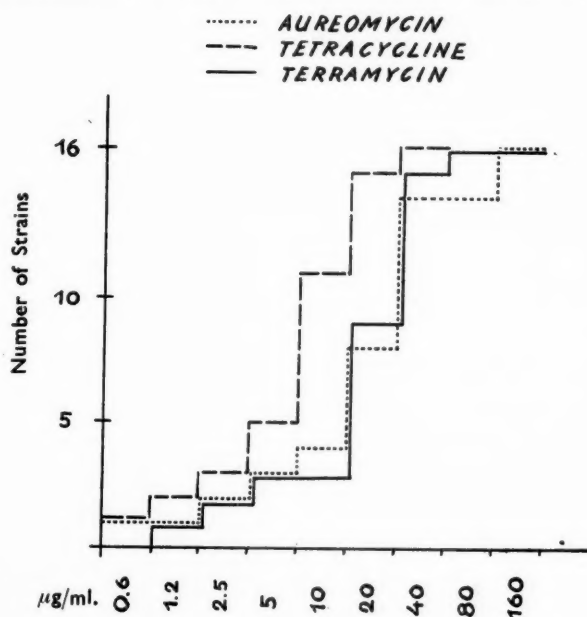
Fig. 5. — The numerical distribution of 16 proteus vulgaris strains by their sensitivity to the above concentrations of aureomycin, tetracycline and terramycin (μg/mk)
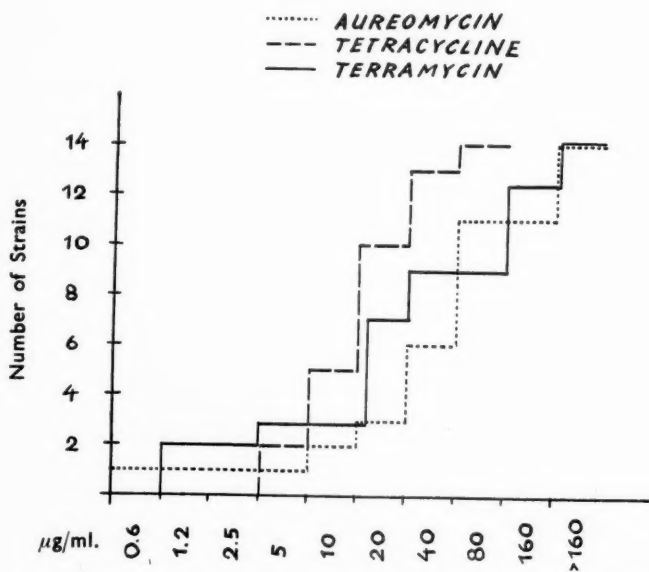
Fig. 6. — The numerical distribution of 14 Proteus rettgeri and Proteus morganii strains by their sensitivity to the above concentrations of aureomycin, tetracycline and terramycin (μg/ml).

TABLE 2

NUMBER AND PERCENTAGE OF PROTEUS STRAINS (OF A TOTAL OF 80) WHICH
WERE RESISTANT TO DIFFERENT ANTIBIOTICS

					Number	Percentage
Resistant to	5	I.U./ml.	Penicillin	....	52	65.0
»	» 20	μg/ml	Dihydrostreptomycin	......	36	45.0
»	» 20	»	Chloramphenicol	....	6	7.5
»	» 10	»	Aureomycin	....	56	70.0
»	» 10	»	Terramycin	....	57	71.3
»	» 10	»	Tetracycline	....	31	38.7

to produce very high blood levels, it is not theoretically inconceivable that, using a high dosage schedule, positive results can be obtained with penicillin in some Proteus infections. As high blood levels generally cannot be reached with tetracyclines, several moderately sensitive strains disappear in this table, and the number of resistant strains becomes marked, especially of strains resistant to aureomycin and terramycin (70 per cent). According to this distribution, too, chloramphenicol seems more effective than all the other antibiotics.

As the composition of the culture medium may greatly affect the sensitivity results, all the sensitivity determinations were repeated with broth. With penicillin, chloramphenicol, terramycin and tetracycline the results were of the same order with both culture media. For aureomycin and dihydrostreptomycin only was there a difference. The diagrams of Figs. 7 and 8 show that the broth reduces the potency of aureomycin and raises that of dihydrostreptomycin against the Proteus mirabilis strains tested. The deviation in the sensitivity results using broth as culture medium was mainly of the same order with the other Proteus types although less marked.

A comparison of our results with those reported by Frank et al. (1) and Potee et al. (3) revealed that our material contained more strains that were sensitive to all the antibiotics used. The difference is most striking for chloramphenicol. Frank reports 30 per cent sensitive to 12.5 μg/ml chloramphenicol, Potee reports 7 per cent; our result was 85 per cent sensitive to 10 μg/ml. Both of them report approx. 10 per cent (our figure, 34 per cent) sensitive to corresponding concentrations of dihydrostreptomycin. Almost all their strains were resistant to penicillin, while our figure was
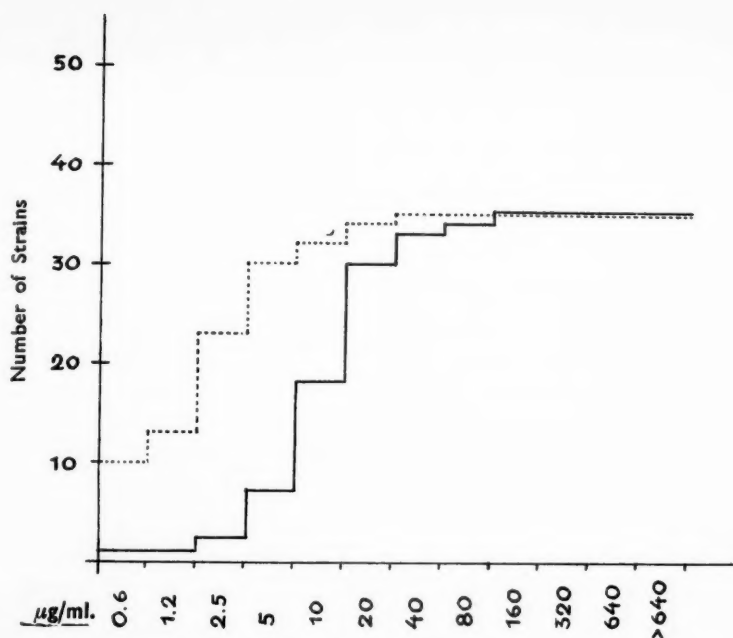
Fig. 7. — Comparison by the twofold serial dilution method of the sensitivities of 50 Proteus mirabilis strains to dihydrostreptomycin, using (a) broth - - - - or (b) modified minimum medium ——— as the culture medium.
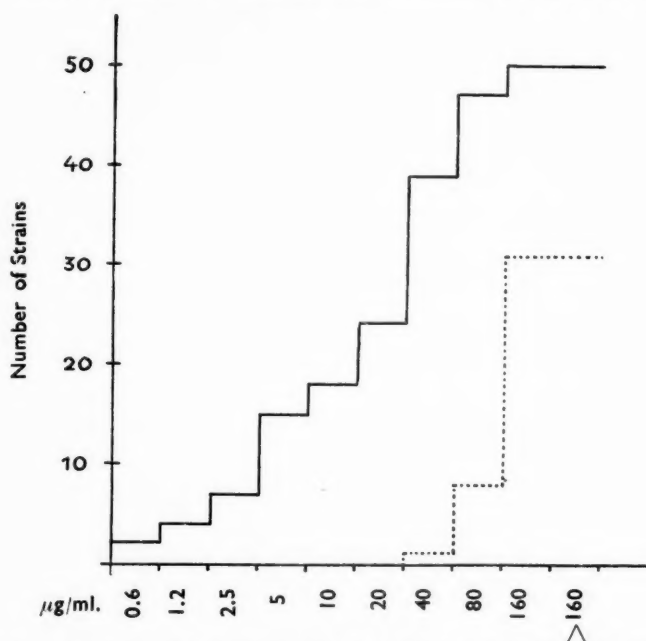
Fig. 8. — Comparison by the twofold serial dilution method of the sensitivities of 50 Proteus mirabilis strains to aureomycin, using (a) broth - - - - - or (b) modified minimum medium ——— as the culture medium.

65 per cent only. In the tetracycline group, Potee reports approx. 10 per cent sensitive to 6.3 μg/ml (our figure, approx. 25 per cent to 5 μg/ml).

SUMMARY AND CONCLUSION

80 strains isolated from urinary tract infections — 50 Proteus mirabilis, 16 Proteus vulgaris, 8 Proteus rettgeri and 6 Proteus morganii — were tested by the twofold serial dilution method for sensitivity to penicillin, dihydrostreptomycin, chloramphenicol, aureomycin, terramycin and tetracycline.

No difference in sensitivity was noted between the Proteus types, and each group reacted similarly to the antibiotics tested. Of all the strains studied, 65 per cent proved completely resistant to penicillin, 71.3 per cent to terramycin, 70 per cent to aureomycin, 45 per cent to dihydrostreptomycin, 39 per cent to tetracycline and 8 per cent to chloramphenicol. It was found that the culture media affected primarily the sensitivities to aureomycin and dihydrostreptomycin.

REFERENCES

1. Frank, P. F., Wilcox, C., and Finland, M.: J. Lab. & Clin. Med. 1950:*35*:205.
2. Palva, I. P., and Kaipainen, W. J.: Ann. Med. exp. et biol. Fenniae 1957:*35*:38.
3. Potee, K. G., Wright, S. S., and Finland, M.: J. Lab. & Clin. Med. 1954:*44*:463.
4. Rhoads, P. S., Billings, C. E., and O'Conor, V. J.: J.A.M.A. 1952: *148*:165.
5. Winkle, S.: Mikrobiologische und Serologische Diagnostik, — Gustav Fischer Verlag, Stuttgart 1955:63.

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OBSERVATIONS ON EXPERIMENTAL FIBRINOLYSIS IN RABBITS

by

JAAKKO ELO, V. J. KÄRKKÄINEN and EERO ESTOLA

In attempts to find substances capable of dissolving a thrombus developed in the blood vessel. in recent years the possibility of using proteolytic enzymes parenterally has been explored. Since 1952, when Innerfield et al. (7, 8, 9) showed that it was possible to give large amounts of trypsin intravenously to animals, provided the enzyme was given highly diluted and very slowly, numerous studies have been made using the same mode of administration. Innerfield's observation, that circulating fibrinogen can be reduced and even eliminated with a large enough amount of trypsin has been confirmed. According to most control investigations, trypsin administered in sufficiently fibrinogenolytic doses is toxic, and furthermore it has usually not been found to have any lytic effect on artificial thrombus. According to some investigations (18) chymotrypsin possesses some thrombolytic effect. The best results have been reached with plasmin and streptokinase, which are also reported to be less toxic (1, 5, 10, 18). In addition to the above, animal experiments have been made with proteolytic enzymes derived from the vegetable kingdom, papain (16) and tyrosinase (19).

In our earlier studies (2) of the fibrinolytic preparate isolated from a mushroom, Tricholoma equestre (T.e. preparate), it was found to resemble plasmin in that while its proteolytic effect seemed to be particularly great on fibrin and fibrinogen, it was clearly

inferior to trypsin in its ability to decompose casein and gelatin. We felt that it would be interesting to compare in vivo the anti-coagulating effects of our T.e. preparate with those of trypsin.

MATERIAL AND METHODS

Fibrinogen: Armour bov. Fr. I
Thrombin: Hoffmann-La Roche
Trypsin: Tryptar (1000 Armour units/mg)

T.e. preparate: Made of crude extract by paper-electrophoretic fractionation and lyophilisation (2). On investigation, the preparate thus obtained was found to contain approx. 15 per cent of protein. The soya bean trypsin inhibitor did not inhibit the effect of the preparate on casein (12). A comparison of the fibrinogenolytic powers of the enzymes showed that 1 mg of T.e. preparate corresponded to 5 mg of Tryptar.

Clotting Time. — According to Lee and White (14), somewhat modified: 10 drops of blood were allowed to drop into 11×100 mm test tubes: the time was recorded from the first drop.

Prothrombin Time. — According to Quick-Lehmann (15), using Ido-kinas tablets by Ferrosan as the source of thrombokinase.

Clot-lysis Reaction. — The tubes used in determining the clotting and prothrombin times were placed in a water bath at $+37°C$; the dissolution of the clot was noted at 24-hour intervals.

Fibrinolysis. — The fibrin plate was made by coagulating 10 cc of fibrinogen solution (0.25 per cent in the veronal buffer of Michaelis at pH 7.0) with 0.2 cc of thrombin (0.25 per cent in the same buffer) in a Petri dish. 0.02 ml of the plasma sample to be studied was pipetted onto the fibrin plate and the dishes were placed in a thermostat at $+37°$ C. The results were read after 24 hours.

Determination of Plasma Fibrinogen. — In the main according to Ratnoff (17), but for the final measurement Goa's (3) micro-biuret method was used. (Beckman Model B spectrophotometer at 330 mμ with filter). Armour bov. plasma albumin was used as the standard.

Determination of Total Plasma Proteins. — 0.1 ml of plasma was pipetted into a 10 ml centrifuge tube, 1.9 ml of saline and 0.5 ml of 5 per cent phosphotungstic acid in 2 N HCl were added, mixed together and centrifuged after 15 min. at 3500 r.p.m. The super-

natant was discarded and the precipitate dried in a vacuum desiccator. 5.0 ml of biuret reagent (4) was pipetted into the precipitate and mixed with a glass rod. The colour was measured after 30 minutes with a Beckman Model B spectrophotometer at 540 mμ. Armour bov. plasma albumin was used as the standard.

Plasma UV Spectrum. -- A 0.1 ml plasma sample from the deep-freeze was diluted with 10 ml of saline. The UV spectrum of the solution, wave range 200—350 mμ, was determined (Zeiss Model PMQ II).

Determination of the Antitryptic Activity of the Plasma. -- The plasma was diluted 1: 100 with saline. The dilution was allowed to react with trypsin for half an hour at $+37°C$. The remaining tryptic activity was then determined spectrophotometrically according to Kunitz (11), using casein as the substrate. A comparison with the trypsin standard indicated how much of the trypsin activity had been inhibited.

TECHNIQUE OF THE EXPERIMENTS

The experimental animals were rabbits weighing 2—3 kg, and the enzymes were administered by infusion lasting 3—3.5 hours.

Infusion Technique. — Under ether anaesthesia, a polythene cannula (»Sterivac») was inserted in the marginal vein of the ear and saline solution from a 100 ml infusion flask was allowed to drip slowly into the vein. After 10—15 minutes, when the rabbit came out the anaesthesia, the dose of enzymes dissolved in a small amount of saline was injected into the infusion flask. The drop rate was adjusted to 6—8 drops per minute.

Taking the Samples. — In a preliminary series of tests the samples were taken by the heart puncture method. As rabbits that had been given enzymes often died in this connection, and the cause of death was verified as cardiac tamponade due to bleeding into the pericardium, it was decided to take the samples from the ear-veins. The free ear lobe was shaved clean, smeared with silicon-containing ointment, and the blood was allowed to drip into the test tube from a puncture in the marginal vein. Provided the blood dripped fast, no essential difference was noted between the sample obtained in this way and another sample taken simultaneously by cardiac puncture. The blood samples were drawn as follows: Sample 1 before the

infusion, Sample 2 immediately at the end of the infusion, Sample 3 one hour after the infusion ended, Sample 4 three hours after the end of the infusion, Sample 5 six hours after the end of the infusion, Sample 6 approx. twenty hours after the end of the infusion.

Handling the Samples. — For determination of the clotting time, 10 drops of blood were taken in 11 × 100 mm test tubes. When clotting was complete the tubes were left in a water bath at +37°C to determine the clot-lysis reaction. In another, graduated tube of the same size, containing 3.8 per cent sodium citrate in water, 4 parts of blood per 1 part of citrate solution were added. This sample was centrifuged immediately on drawing for 20 minutes at 3 500 r.p.m., and the plasma was transferred into another siliconized tube. Aliquots of 0.5 ml and 2 × 0.1 ml were transferred from it immediately into different tubes for the determination of fibrinogen, total proteins and UV-spectrum, and all the tubes were placed in the deep-freeze at —20°C. The day after, in addition to the tests above, the prothrombin index was measured as well as the anti-tryptic and fibrinolytic effects.

TOXICITY

Individual variations between the rabbits were considerable, but all of them tolerated without complications the dose of 10 mg/kg of Tryptar and 2 mg/kg of T.e. preparate. With higher doses toxic reactions of varying degree were noted. A milder manifestation was indicated by the rabbit's ears turning cyanotic and cold, and on drawing the sample the blood dripped slowly. In a severe reaction the rabbit died after intense convulsions. Autopsy findings included typical stress changes like blood-filled inner organs, parenchymal degeneration, cytolysis and in some cases even necroses in the adrenals. Extensive haemorrhages were never encountered.

RESULTS ·

Although in some instances the rabbits recovered even after their blood had become completely uncoagulable, the usual result of such a strong stress was death. The amounts of enzyme that the rabbits tolerated without any toxic reactions on no occasion caused complete fibrinogenolysis. However, distinctly different reactions from the rabbits receiving saline only were noted. The results

reported below refer to a series of experiments in which five rabbits were given 100 ml of saline only in a 3-hour infusion, and five were given 10 mg/kg of Tryptar and five 2 mg/kg of T.e. preparate, in an identical volume of saline in the same time. All the animals tolerated the infusion without demonstrable toxic reactions.

Clotting Time. — The clotting times of the control rabbits varied from 2—6 minutes, the maximum times of those treated with enzymes from 6—9 minutes. A prolonged clotting time was usually only noted in Samples 2 and 3 (see above). Determination of the exact clotting time, especially in the presence of proteolytic effect, is fairly difficult. For a certain level of reliability the blood must drip fast.

Prothrombin Index. — Saline infusion usually produced a small drop in the prothrombin index, the lowest value being 90 per cent (Sample 2). In Sample 3 the index regularly returned to 100 per cent. The maximal effect of Tryptar infusion was seen in Sample 2 as well, the lowest value being 65 per cent. The index was still at a lowered level in Sample 5 (80—90 per cent); by Sample 6, however, taken next morning, it was normal. The effect of T.e. preparate infusion differed from that of Tryptar in that it lasted longer, and the maximum came in Sample 3. The lowest index value was 30 per cent. 20 hours after the infusion the values had not yet normalized (index approx. 90 per cent). (Figs. 1—3).

Clot-lysis Reaction. — The spontaneous lysis of the whole blood coagel turned out to be a specific and fairly serviceable indicator of the fibrinolytic process. Samples taken in connection with saline infusion never dissolved spontaneously when left in a water bath at $+37°C$. This was true with Tryptar also in the dosage employed. But rabbits treated with T.e. preparate regularly showed one or more samples that had dissolved, and in many instances the phenomenon was noted even in a sample drawn 3 hours after the infusion. (Figs 1-3).

Similarly, spontaneous lysis of the plasma coagel obtained in the determination of the prothrombin time could only be noted in rabbits that had been given T.e. preparate; it was not, however, always complete and was limited to fewer samples. Both these clot-lysis reactions reached their maximum value after an incubation of 48—72 hours. (Figs. 1—3).

No fibrinolysis in the plasmas studied could be shown by the fibrin plate method.

Determination of Plasma Fibrinogen. — Control samples, stored for 24 hours in the deep-freeze at —20°C, very often showed after thawing a weak profibrin coagel. It was usually not seen in samples taken after the enzyme infusion. It did not however interfere with the fibrinogen determination as it coagulated in the presence of thrombin together with the soluble fibrinogen. A study of parallel samples showed that it contained 15—25 per cent of the fibrinogen in the sample.

In control samples taken before the infusion the fibrinogen content ranged from 140—392 mg per cent. Saline infusion produced a distinct drop in the fibrinogen concentration, and this was seen most markedly in the sample taken 2 hours after the infusion (an average of 76 per cent of the control value). By the next day the value had returned to its former level. The maximal effect of Tryptar came in the sample taken 3 hours after the infusion (66 per cent), but in contrast to the former the value in most experiments still remained at a lowered level in the sample taken the next day. The T.e. preparate reached its maximal effect 6 hours after the infusion (57 per cent), and on the next day the fibrinogen content was regularly still below the initial value.

Figs. 1—3 show the prothrombin indices, fibrinogen amounts and the results of the clot-lysis reaction of three experiments. In these experiments an extra sample was taken during the infusion.
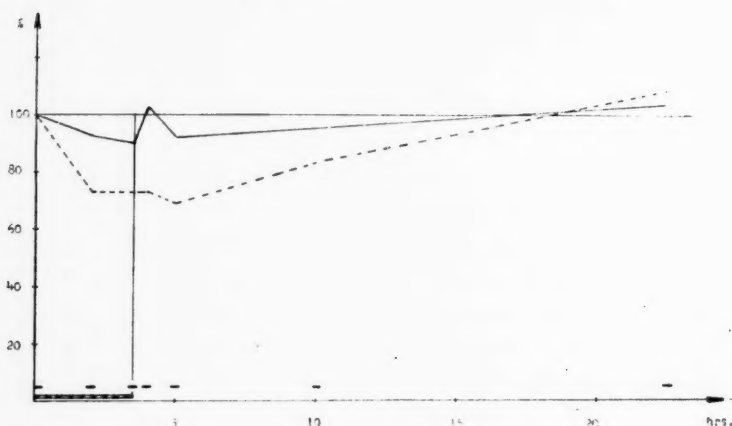
Fig. 1
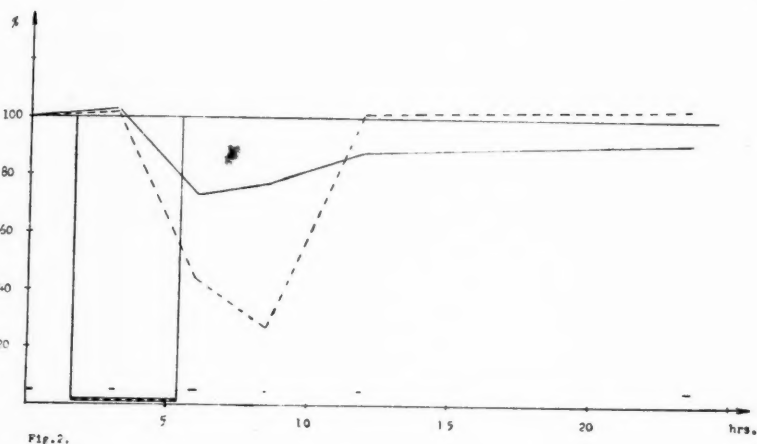
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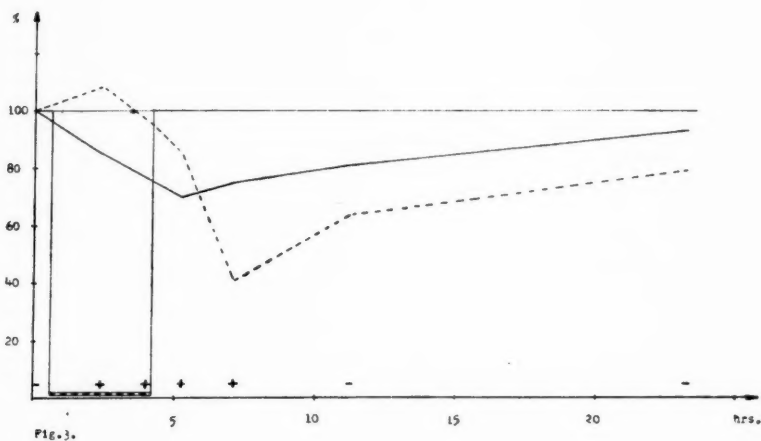
Fig. 3.

Figs. 1—3. — Typical curves from three experiments showing the variations in the prothrombin indices (——) and the amounts of fibrinogen (- - -). Ordinate: Percentage values of the control sample. Abscissa: Time beginning from the taking of the control sample (hours). Fig. 1. — Infusion of saline (100 ml). Fig. 2. — Infusion of Tryptar solution (10 mg/kg in 100 ml of saline). Fig. 3. — Infusion of T.e. preparate solution (2 mg/kg in 100 ml of saline). Infusion time: ▮▮▮▮ Result of the clot-lysis reaction: + or —

Determination of Total Plasma Proteins. — In the control samples taken before the infusion the total protein varied 5.00—5.80 per cent. Saline infusion caused a clear drop in the total plasma protein, the maximal effect occurring 2 hours after the infusion (an average of 80 per cent of the control value). This protein dilution seemed to be somewhat more marked with saline than it was in rabbits given an equal volume of enzyme solution (Fig. 4).
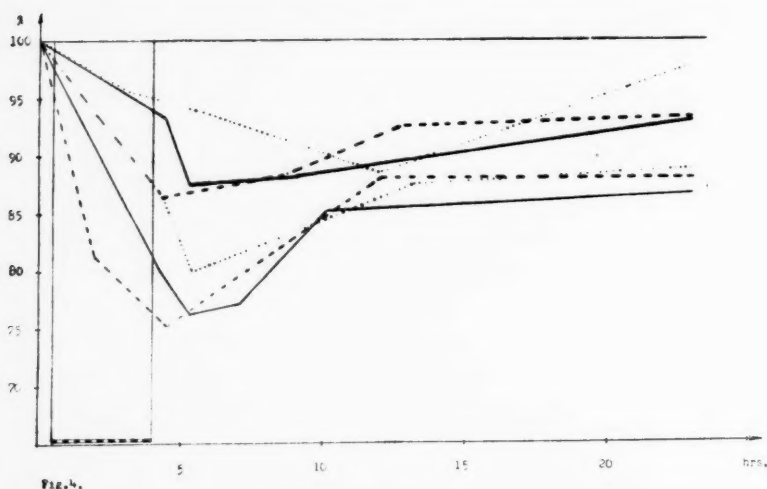
Fig. 4. — The variations in total plasma protein during the experiments. Ordinate: Total plasma protein in percentage values of the control sample. Abscissa: Time as in Fig. 1. The results of all five experiments with saline fall between the lines marked: — — — Experiments with Tryptar between: . . . . . . Experiments with T.e. preparate between: ———

The UV Spectrum of the Plasma. — The most definite changes were observable in the range 250—290 mμ. The extinction at 278 mμ of the samples taken in the initial stage of the experiment declined in proportion to the total protein value. In the saline infusion group this went on throughout the experiment, while for the animals given the enzyme solution the extinction at 278 mμ began to rise in proportion to the total protein values 3 hours after the infusion. The change was greatest in the last sample, taken 20 hours after the infusion (Fig. 5).

The Antitryptic Activity of the Plasma. — Saline infusion regularly produced a slight drop in the antitryptic activity of the plasma.
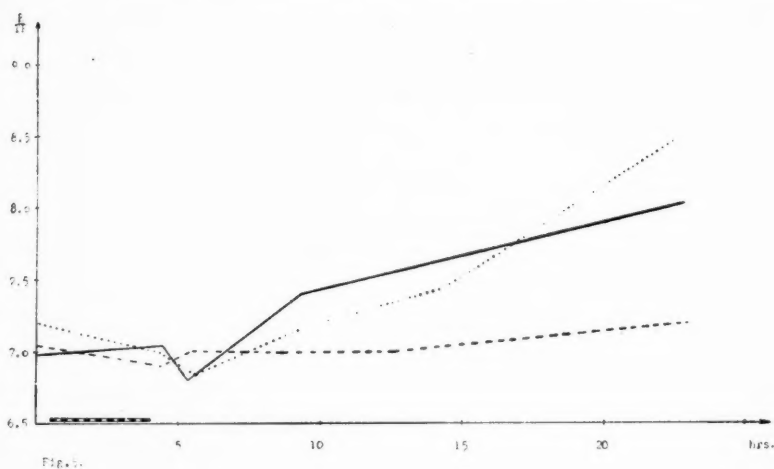
Fig. 5. — The variations in the proportion $\dfrac{E}{TP}$ during the experiment. (E = $10^2 \times$ the extinction value at 278 mμ from the UV-spectrum; TP = amount of total protein in g/100 ml measured with the biuret method). Ordinate: $\dfrac{E}{TP}$. Abscissa: Time as in Fig. 1. Symbols for the different infusions as in Fig. 4.
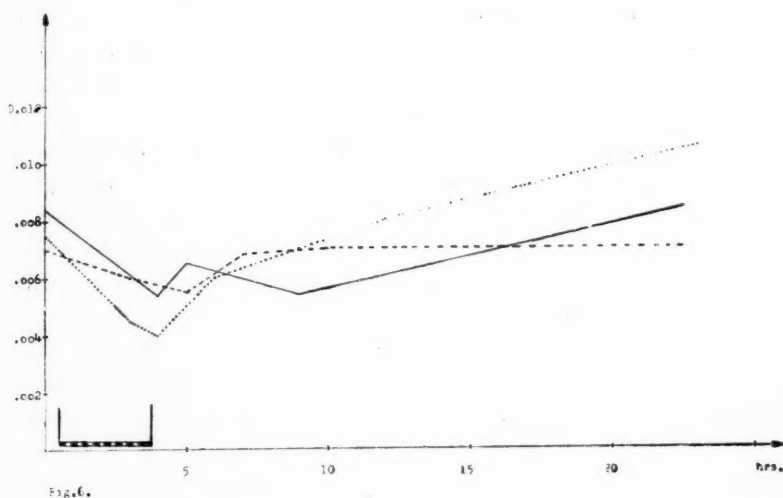
Fig. 6. — The variations in antitryptic activity in three experiments. Ordinate: Mg of trypsin inhibited. Abscissa: Time as in Fig. 1. Symbols for the different infusions as in Fig. 4.

The effect of enzyme infusions differed from the saline control only in that the antitryptic activity of the trypsin-treated rabbits was usually above the initial value in the sample taken 20 hours after the infusion (Fig. 6).

<div style="text-align:center">DISCUSSION</div>

Previous investigations into the use of trypsin in animal experiments have shown that toxicity is greatly dependent on the concentration and rate of the infusion given. Innerfield et al. (8) used for rabbits trypsin quantities of up to 125 mg/kg (125.00 u. Tryptar) and for dogs 100 mg/kg (100.000 u.). Taylor et al. (20) reported a 50 per cent lethality in rabbit experiments with approximately the same mode of administration and a dosage of 110 mg/kg (200.000 u. Enzar). Hardy et al. (6) found that, for dogs, the »upper limit of safe dosage is less than 50 mg/kg» (50.000 u. Enzar). In our preliminary tests we found that the toxicity limit of the trypsin used was as low as 10 mg/kg (10.000 u.). The quantity of T.e. preparate that in in vitro experiments had proved of equal fibrinogenolytic activity, 2 mg/kg, proved to be similar in toxicity also. Using these quantities, we were able to carry out the experiments on non-anaesthetized animals without observing any signs of toxicity in the behavior of the animals. In proportion to the small amount of enzymes given, the changes produced by the infusion were also relatively small; however, distinct differences from the effect produced by saline infusion were observable.

From determinations of the prothrombin index and fibrinogen it was found that the effect produced by trypsin was of shorter duration than that of T.e. preparate, evidently due to the inhibitory effect of plasma on the former. Otherwise no major difference was noted between the enzymes. The »clot-lysis» reaction, the spontaneous dissolution of the blood sample coagel at $+37°$ C, turned out to be a specific indicator of proteolysis. According to earlier investigations, this reaction is very sensitive in animal experiments where streptokinase and plasmin are used, and is observable in less than 30 minutes. With trypsin, Sherry et al. (18) failed to achieve lysis of the blood sample in dog experiments using a dosage of 16—33 mg/kg, if the sample had coagulated after the infusion. Laufman et al. (13), giving 100.000—200.000 tryptic units to dogs weighing 10—15 kg (i.e. 10.000—20.000 u./kg), obtained a

partial lysis, within a few hours even, and a complete lysis with 250.000 u. in 15 minutes. Hardy, with his above-mentioned dosage, noted spontaneous lysis of blood coagel in two instances only. In the present experiments we never obtained the clot-lysis reaction with the amount used, 10.000 u./kg. of trypsin, in spite of an observation period of several days. But with T.e. preparate it was usually achieved in samples taken 1—3 hours after the infusion. However, the reaction was slower than those reported above: it was not noted until after 12—24 hours of incubation, reaching its maximum after 48—72 hours of incubation. According to Laufman, a coagel formed during trypsin infusion, because of the enzyme it contains, dissolves more readily than a normal coagel: in spite of a positive clot-lysis reaction he observed no lysis in a thrombus formed prior to infusion.

The antitrypsin study showed that during trypsin infusion even considerable antitryptic activity remains in the plasma, a fact that accounts for the weak effect of trypsin.

The measurement of the UV absorption spectrum revealed a phenomenon that may also be due to proteolysis. It was found that UV-extinction at 278 mμ in the samples taken in the initial stages of the experiment followed the total protein values whereas in the samples taken in the final stages it gave clearly higher values. The degree of the proteolysis possibly involved here remained obscure.

The total plasma protein values of the samples taken after the infusion decreased less in the enzyme group than in the control group. The explanation may be that even this small quantity of enzymes may have produced in the animals a slight subclinical shock condition with hemoconcentration, although no outward signs were visible.

The varying data reported in the literature on the toxicity of the different enzyme preparations suggest that the toxic phenomena are due in part at least to impurities. The toxicity of the split products is decisive from the point of view of parenteral administration of the enzymes

SUMMARY

A comparison was made of the effects on rabbits of trypsin and a proteolytic enzyme of fungal origin. Tricholoma equestre preparate, administered by slow drip infusion. The animals tolerated well the

doses, 10 mg/kg of trypsin and 2 mg/kg of T.e. preparate. A higher dosage produced toxic reactions of various degrees. The changes produced by the infusion in the clotting time, prothrombin index, antitryptic activity and plasma fibrinogen, total proteins and UV-spectrum were studied. With the dosage employed, the changes were small; they were more persistant after T.e. preparate than after trypsin. The spontaneous lysis of a blood sample coagel proved a specific, though not particularly sensitive method of observing the proteolysis produced by enzyme infusion.

REFERENCES

1. CLIFFTON, E. E., GROSSI, C. E., and CANNAMELA, DOLLY, A.: Ann Surg. 1954:*139:*52.
2. ESTOLA, E., ELO, J., and KÄRKKÄINEN, V. J.: Ann. Med. Exp. Biol. Fenniae 1955:*33:*384.
3. GOA, J.: Scand. J. Clin. Lab. Invest. 1953:*5:*218.
4. GORNALL, A. G., BARDAWILL, CH. J., and DAVID, M. M.: J. Biol. Chem. 1949:*177:*751.
5. GROSSI, C. E., CLIFFTON, E. E., and CANNAMELA, DOLLY, A.: Blood 1954:*9:*310.
6. HARDY, E. G., MORRIS, G. C., and DE BAKEY, M. E.: Surg. Gyn. Obst. 1955:*100:*91.
7. INNERFIELD, I., SCHWARZ, A. W., and ANGRIST, A.: Bull. New York Acad. Med. 1952:*28:*537.
8. INNERFIELD, I., SCHWARZ, A. W., and ANGRIST, A.: J. Clin. Invest. 1952:*31:*1049.
9. INNERFIELD, I., ANGRIST, A., and BENJAMIN, J. W.: Gastroenterology 1952:*20:*630.
10. JOHNSON, A. J., and TILLETT, W. S.: J. Exper. Med. 1952:*95:*449.
11. KUNITZ, M.: J. Gen. Physiol. 1947:*30:*306.
12. KÄRKKÄINEN, V. J., ESTOLA, E., and ELO, J.: to be published.
13. LAUFMAN, H., and ROACH, H. D.: A.M.A. Arch. Surg. 1953:*66:*552.
14. LEE, R. I., and WHITE, P. O.: Am. J. M. Sc. 1913:*145:*495.
15. LEHMAN, J.: Nord. Med. 1941:*12:*3192.
16. QUICK, A.: Fed. Proc. 1956:*15:*332.
17. RATNOFF, O. D., and MENZIE, C.: J. Lab. Clin. Med. 1951:*37:*316.
18. SHERRY, S., TITCHENER, ANTOINETTA, GOTTESMAN, L., WASSERMAN, P., and TROLL, W.: J. Clin. Invest. 1954:*33:*1303.
19. SIZER, I. W.: Science 1952:*116:*275.
20. TAYLOR, A., OVERMAN, R. S., and WRIGHT, I. S.: J.A.M.A. 1954:*155:*347.

STUDIES ON THE LOCAL REACTIONS OF THE SKIN TO CHEMICAL COMPOUNDS

by

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Our aim was to study the skin reactions caused by aliphatic alcohols, as the rules of their bactericidal and bacteriostatic activities are well known (1). The activity of alcohols increases with the number of carbon atoms until the poor solubility of the higher alcohols masks their effect. The primary alcohols are more active than the secondary ones and the latters more active than the tertiary ones. The branched chain primary alcohols are somewhat less active than the normal primary alcohols.

As a basis for comparison, we injected 10 mg and 35 mg of each alcohol solved or emulged in 0.1 ml of water or paraffin oil intracutaneously in the dorsal skin of rabbits. The following tables indicate the size of the skin reaction as a product of two diameters. The measurements were performed 24 hours after the injection. All the alcohols to be compared were injected in the same animal and simultaneously. The figures in the Table 1 represent mean values of four experiments.

Even if the injections are not exactly repeatable and the rabbits react individually, there is but little doubt that the size of the reaction increases with the length of the carbon chain and drops then with rapidly decreasing solubility of alcohols. The shift of the maximal reaction from butyl- to amylalcohol with 35 mg and

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TABLE 1

THE SIZE IN SQ MM OF LOCAL SKIN REACTIONS AFTER INTRADERMAL INJECTIONS
OF NORMAL ALIPHATIC ALCOHOLS (FOUR RABBITS)

Amount Injected	Methyl-	Ethyl-	Propyl-	Butyl-	Amyl-	Hexyl-	Octyl-	Heptyl-
35 mg in water	9	47	75	83	74	60	40	33
10 mg	0	0	6	11	19	18	14	14
35 mg in paraffin	3	2	25	102	53	38	17	6
10 mg oil	1	2	5	9	40	21	—	11

10 mg, respectively, is noteworth and may be connected with
solubility differences. Accordingly, the pattern is very similar to
the bactericidal and bacteriostatic effects of these alcohols.

TABLE 2

THE SIZE IN SQ MM OF LOCAL REACTIONS AFTER INTRADERMAL INJECTIONS OF
PRIMARY, SECONDARY AND TERTIARY ALCOHOLS (EIGHT RABBITS)

Amount Injected	Propyl Alcohol		Butyl Alcohol			Amyl Alcohol		
	Prim.	Second.	Prim.	Second.	Tert.	Prim.	Second.	Tert.
10 mg	73	57	99	94	43	115	95	66
35 mg	19	13	38	30	14	47	36	36

The reactions obtained with primary, secondary and tertiary
alcohols (Table 2) seem roughly to follow the same rules as
did the bactericidal and bacteriostatic activities of these alcohols.
The primary alcohols were little more active than the secondary
ones and the tertiary alcohols were in general least active. How-
ever, the individual rabbits reacted frequently in an adverse way.

The bactericidal and bacteriostatic effects of isoalcohols are
definitely below that of the normal primary alcohols. When com-

TABLE 3

THE SIZE IN SQ MM LOCAL OF SKIN REACTIONS AFTER INTRADERMAL INJECTIONS
OF NORMAL AND ISO-(BUTYL AND AMYL) ALCOHOLS (EIGHT RABBITS)

Amount Injected	Butyl Alcohols		Amyl Alcohols	
	Normal	Iso-	Normal	Iso-
35 mg	98	98	55	102
10 mg	40	29	28	45

paring the skin reactions caused by them such a difference is not evident (Table 3).

The skin reactions caused by isoamylalcohol are definitely larger in size than those caused by the normal amylalcohol. There is no significant difference in the solubility of these compounds. Accordingly, we have here an exception of the main parallelism of the skin reactivity and bactericidal activity of aliphatic alcohols.

SUMMARY

Aliphatic alcohols studied cause skin reactions when injected intradermally. The size of the reaction seems to increase with the number of carbon atoms. Butyl and amyl alcohols gave mostly the largest reactions. The weaker reactions caused by hexyl, heptyl and octyl alcohols are supposed to be due to their poor solubilities. Secondary alcohols seem to be somewhat less active than the primary ones, but more active than the tertiary alcohols. So far the rules as similar to the bacteriostatic and bactericidal activities of aliphatic alcohols and to their ability to cause skin reactions in rabbits. Isoamyl alcohol only did not conform to the general rule causing unexpectedly strong skin reactions.

REFERENCE

1. Kokko, U. P.: Arch. f. Hyg. u. Bakt. 1939:*122*:44.

OBSERVATIONS ON THE DONAGGIO REACTION

by

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The Donaggio reaction has been mentioned in the literature since 1933, when A. Donaggio published a study on the peculiar »inhibition phenomenon» in the urine of malaria patients. The precipitation of the aniline dye thionine by means of ammonium molybdate does not occur, and the solution preserves its violet colour. By calling the reaction negative is meant that thionine sinks as a precipitate on the bottom of the test tube and the liquid remains colourless. Urine has mainly been used as the test substance, but also experiments made with spinal fluid have resulted in positive reactions.

In various fever conditions the urine has been found to be Donaggio positive (1, 2, 3, 5, 6, 7, 8, 9, 17).

Positive reactions have been mentioned also in connection with tumors (5, 9).

Some quite interesting observations have been made concerning increased Donaggio positiveness of urine due to muscular action. Attention was drawn to the fact that the increase in the urine of a well trained person was smaller than that of a less trained one after the same amount of work. Therefore the reaction was found suitable for evaluating the results of physical training (4, 10, 11).

The diet also seems to affect it a protein diet increasing and a carbohydrate diet decreasing the reaction (19). Keeping rats on starvation decreases the Donaggio reaction in their urine (20). Normally the urine of rats, as well as of any other carnivores, is positive, whereas that of herbivores is negative (15).

The substance that causes the reaction has been isolated by precipitating it with alcohol (14, 15). The preparation isolated by following approximately the same principle has been analysed rather thoroughly (12, 13). It has been found to contain glucosamine, but hyaluronidase does not affect it. The substance reduces first after a prolonged acid hydrolysis (15). Concerning the dialysis of the Donaggio-positive substances the results were quite opposite (3, 21). Chromatographically the Donaggio positive substance has been identified to be a mucoprotein (16).

The origin of the colloidal substances in the urine seems to be the mucoproteins of the plasma (10, 18).

The purpose of the present work was to investigate the method of performance of the reaction and the effect of the salt concentration and the pH on the positiveness of the reaction. In addition, an attempt was made to explain the Donaggio reactions of heparin, chondroitinsulphuric acid, hyaluronic acid and of the mucopolysaccharide preparation isolated from the urine, as well as of certain fluids of the organism.

METHOD

In the experiments concerning the reaction the following method was used: Urine or some other sample is acidified with acetic acid, boiled for 1—2 minutes, cooled and filtered. The filtrate, the pH of which is brought to 5, is measured in increasing amounts into test tubes (e.g. 0.2—0.4—0.6 ml, a.s.o.), distilled water is added ad 2 ml, after which 1 ml of 0.1 per cent thionine solution and then 2 ml of 4 per cent ammonium molybdate are added. Before the molybdate is added the tubes should be shaked to mix the thionine evenly in the liquid. The series of test tubes is kept still for 24 hours. When evaluating the results the first tube in which the thionine has entirely remained in solution or in which the intensity of the colour has reached a maximum from which it does not increase any further is considered positive.

The values of the Donaggio reaction given here refer to the number of ml's of the tested substance which is sufficient to bring about a positive reaction.

It is preferable to perform the reaction without addition of the water if the positive reaction does not occur with 2 ml. This was

actually the procedure in cases of this nature. The results thus obtained are not quite directly comparable to those obtained after the addition of the water, as the reaction is slightly more sensitive with a larger quantity of water.

Another method for the quantitative determination of the reaction would be the use of always the same amount of urine or other substance and increasing amounts of thionine. The reaction would then be the more positive the greater the inhibition of the precipitation of thionine (table 1).

A photometre cannot be used for the quantitative measurement of the reaction, as the intensity of the colour is not in direct proportion to the amount of substance needed to bring about the reaction.

FACTORS AFFECTING THE REACTION

Whey was used in most of these experiments, as it is very positive for the Donaggio reaction.

1. *Amount of Thionine.* — The reaction is the more sensitive the less there is thionine (table 1). Instead of 1 ml, 0.5 ml of thionine can be used as well, but the colour produced by 0.1 ml may be too faint to enable determination of the reaction.

TABLE 1

DEPENDENCE OF THE SENSITIVITY OF THE REACTION ON THE AMOUNT OF THIONINE. D.R. = AMOUNT OF WHEY NEEDED TO PRODUCE THE REACTION. DISTILLED WATER AD 1 ML WAS ADDED TO THE THIONINE. IN EACH TUBE THE AMOUNT OF MOLYBDATE WAS 2 ML. THE SENSITIVITY OF REACTION SEEMS TO BE INVERSELY PROPORTIONAL TO THE AMOUNT OF THIONINE

Amount of Thionine, ml	D.r. of Whey, ml
1.0	0.35
0.5	0.175
0.1	0.030

2. *Amount of Molybdate.* — It was found that the best proportion between the amounts of thionine and molybdate is 1: 2. In case the proportion is greater the sensitivity will be redused. On the other hand, again, there is no reason to use too much molybdate even though it would not affect the reaction.

TABLE 2

EFFECT OF THE AMOUNT OF MOLYBDATE ON THE REACTION. THE NUMBERS IN THE TABLE REFER TO THE D.R. OF WHEY. DISTILLED WATER WAS ADDED TO EACH TUBE TO KEEP THE VOLUME CONSTANT

Thionine ml	Molybdate, ml				
	0.2	0.5	1.0	2.0	3.0
0.5	0.2	0.15	0.125	0.125	
1.0		0.8	0.5	0.25	0.25

3. *Effect of pH.* — a) By using the same amount of test substance and different pH values and by measuring the extinctions with a photometre the results given in fig. 1 were obtained.
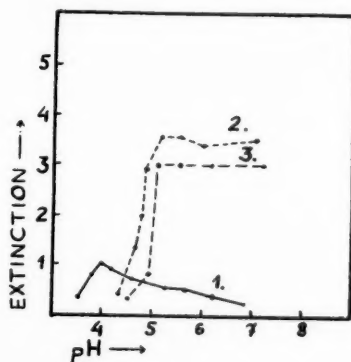
Fig. 1. — Diagram showing the effect of pH on the Donaggio reaction. Intensity of colour measured by photometre. Curve 1: 2 ml of urine (slightly positive) in each tube. In this case the optimum pH seems to be 4.0. Curve 2: 0.4 ml of saliva in each tube. The intensity of colour remains constant above pH 5. Curve 3: 0.4 ml of whey. The intensity of colour is constant above about pH 5.

The intensity of colour in the reactions of whey and saliva at above pH 5 was constant. At lower pH values it went down steeply.

Slightly positive urine had a distinct point of optimum varying between pH 4 and 5 for different urines. The optimum of the preparation isolated from urine according to F. Tayeau (15) was pH 4.7.

b) The reaction was evaluated as described above by means of test tube series at different pH values. The reactions of whey and rat urine were constant at above pH 5 and declined steeply at lower pH's (fig. 2).
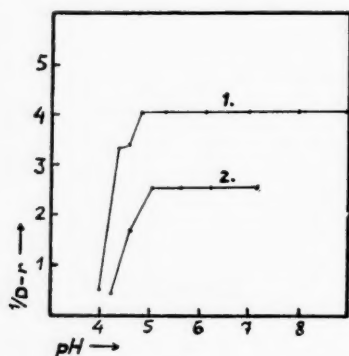
Fig. 2. — Diagram showing the reaction determined with test tube series at different pH's. D-r. = Amount of test substance (ml) needed to bring about a positive reaction. Curve 1: Whey. The reaction is constant at above pH 4.8. Curve 2: Rat urine. The reaction is constant at above pH 5.0.

We used the pH value of 5.0—5.5, as the reaction appeared to be most sensitive at this range.

4. *Effect of Salt Concentration.* — a) To 1 ml of saliva various amounts of sodium chloride were added to make concentrations of 0—10 per cent. The extinction was measured with a photometre. The intensity of colour went clearly down as the salt concentration in creased (fig. 3).
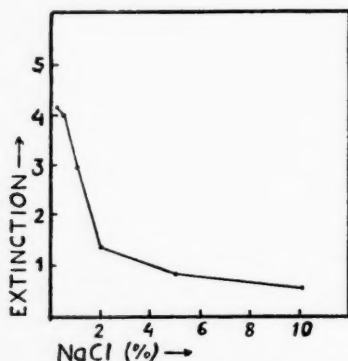
Fig. 3. — Diagram showing the effect of salt concentration on the reaction. Intensity of colour was measured by a photometre. Saliva, to which NaCl was added to make concentration of 0—10 %, was used in the test. The amount of saliva in each case 1 ml. With increasing concentrations the extinction declined steeply due to precipitation of thionine caused by NaCl.

If more than 1 per cent of sodium chloride was added, the thionine partly precipitated already before the addition of molybdate This is the reason why the colour intensity decreases.

b) In evaluating the reaction by means of test tube series, in which NaCl had been added to the whey to make concentrations of 0—5 per cent, it was found that the reaction did not decrease when this method was used (table 3). In concentrations of 1—5 per cent NaCl some of the thionine precipitated already before the addition of molybdate. In the series using 5 per cent NaCl the reaction was more sensitive and the colour turned from violet to blue.

TABLE 3

EFFECT OF SALT CONCENTRATION ON THE DONAGGIO REACTION, USING TEST TUBE SERIES

NaCl, %	D.r., ml
0	0.4
1	0.4
2	0.4
3	0.4
5	0.3

5. *Effect of Heating and Dialysis on the Donaggio Reaction.* — a) When evaporating whey on a boiling water bath until there is no liquid left it was found that the reaction decreased as shown in Table 4.

TABLE 4

DONAGGIO REACTION OF WHEY DETERMINED BEFORE AND AFTER EVAPORATION. WHEY WAS EVAPORATED UNTIL NO LIQUID WAS LEFT, DISTILLED WATER WAS ADDED UP TO THE ORIGINAL VOLUME, AND THE REACTION WAS DETERMINED AGAIN. AFTER THIS THE EVAPORATION, ADDITION OF WATER AND THE DETERMINATION OF THE REACTION WAS REPEATED

	D.r., ml
Before evaporation	0.25
After 1st evaporation	0.30
After 2nd evaporation	0.40

b) When dialysing the whey for 3 days against distilled water, with change of water every 24 hours, the Donaggio reaction of the whey declined from 0.35 ml to 0.50 ml. At the same time a white precipitate occurred in the whey.

The dialysis was performed also without changing the outer liquid. After 10 days the Donaggio reaction of both the inner and the outer liquid was determined (table 5).

TABLE 5

REACTIONS OF THE INNER AND OUTER LIQUIDS BEFORE AND AFTER THE DIALYSIS OF WHEY. THE INCREASE IN THE VOLUME OF THE INNER LIQUID HAS BEEN ADJUSTED. THE OUTER LIQUID DID NOT GIVE A POSITIVE DONAGGIO REACTION

	Amount	D.r. before	After Dial.
Inner liquid	100 ml	0.35	0.40
Outer liquid	200 »	—	—

In connection with the latter dialysis, some white precipitate was produced in the inner liquid, as in the preceding experiments. The outer liquid was still entirely negative at 5 ml, which indicates that the Donaggio-positive substance had not been dialysed at least in considerable amounts.

DONAGGIO REACTION OF CERTAIN SUBSTANCES AND LIQUIDS

By using the test-tube method described above, the following Donaggio reaction values were obtained:

Human urine .... 0.8 —10 ml
Rat urine .... 0.4 »
Saliva .... 0.2 —0.7 »
Whey.................................. 0.25—0.4 »
Gelatine 0.2% .... 0.4 »
Starch 3% .... 7.0 »
Cow serum (a single test) .... 0.5 »

Preparations isolated by alcoholic precipita-
tion (F. Tayeau):

Urine preparation 0.2% .... 0.4 »
Whey preparation 0.2% .... 0.3 »

Mucopolysaccharide isolated from the urine

with NaCl:0.15% .... 1.4 ml

Heparin 0.25% .... 2.5 »

Chondroitinsulphuric acid (0.5%) and hyaluronic acid (0.2%) were still negative at 0.5 ml. They precipitated thionine already before the addition of molybdate. These substances as such could not therefore be the cause of the reaction in the biological concentrations.

DISCUSSION

Several different substances give a positive Donaggio reaction. For certain mucopolysaccharides the reaction is negative at least in the biological concentrations. This does not, however, prevent the presumption that the protein compounds of these substances would be some of the factors bringing about the reaction.

The boiling of the liquids before they are measured into the test tubes is necessary, as in most cases the results will otherwise be negative or vary indefinitely. For instance an unboiled gelatine solution gives a negative reaction. Although no precipitation takes place during boiling, the solution turns positive. If, again, milk is made acid but the filtrate is not boiled, the latter part of the test tube series will be negative, while the tube indicating the border-line of positivity in the first part will vary somewhat. In the first part of the series about 2—3 tubes will be found to have retained their colour. Protein thus seems to disturb the reaction. On the other hand, the negativeness of the gelatine solution before boiling can hardly be explained likewise. It could be presumed that when gelatine is boiled, some substance that reacts Donaggio-positively is liberated. Vigorous boiling, however, reduces the reaction and some of the substance is obviously destroyed. Inactivation may also be a cause of decrease in the reaction in connection with dialysis (i.e., the formation of the precipitate).

The test tube series method used above seems to be sufficiently accurate for the quantitative measurement of the reaction. The salt concentrations of the biological liquids do not have a disturbing influence. According to the literature the clinical importance of the Donaggio reaction seems to be rather insignificant.

SUMMARY

It was our purpose to study methods of procedure in the Donaggio reaction and the factors affecting the reaction. The quantitative determination was performed by means of series of test tubes. In addition to urine and spinal fluid, the reaction was found to be positive also in saliva, whey and gelatine. The sensitivity was inversely proportional to the amount of thionine. Before carrying out the measurement it is well to adjust the pH of test substance to pH 5. The salt concentration was found to have no effect when the above method was used. Heating and dialysis reduced the reaction.

REFERENCES

1. Bozzi, E.: Boll. soc. ital. biol. sper. 1936:*11*:168.
2. Colombati, S.: Boll. soc. ital. biol. sper. 1936:*11*:780.
3. Donaggio, A.: Boll. soc. ital. biol. sper. 1933:*8*:1459.
4. Fabre, R., Rougier, G., Harramburu, S., and Allard, P.: Compt. rend. soc. biol. 1947:*141*:74.
5. Kano, M.: Med. J. Hirosaki 1950:1 (2):30 and *1* (3):46.
6. Kano, M.: Med. J. Hirosaki 1951:*2*:131, 252.
7. Kano, M.: Med. J. Hirosaki 1952:*3*:1.
8. Lanfranchi, A., and Foresti, C.: Boll. soc. ital. biol. sper. 1936:*11*:777.
9. Mallone, T.: Biochim. terap. sper. 1935:*22*:600.
10. Nakayama, T.: Igaku to Seibutsugaku (Med. a. Biol.) 1953:*29*:5, 95.
11. Saito, H., and Urano, H.: Igaku to Seib. (M.a.B.) 1952:*25*:13.
12. Shimokawa, M.: Igaku to Seib. (M.a.B.) 1951:*20*:150.
13. Shimokawa, M.: Igaku to Seib. (M.a.B.) 1952:*22*:139.
14. Sollazzo, G., and Cortese, V.: Boll. chim. farm. 1935:*74*:625.
15. Tayeau, F.: Tunisie Med. 1951:*39*:664.
16. Tayeau, F., Biserte, G., Montreuil, J., Marquevelk, S.: Compt. rend. 1953:*237*:208.
17. Ugami, H.: Igaku to Seib. (M.a.B.) 1952:*24*:41.
18. Utsuno, Y.: Igaku to Seib. (M.a.B.) 1952:*25*:29, 233.
19. Utsuno, Y.: Igaku to Seib. (M.a.B.) 1953:*27*:23.
20. Utsuno, Y.: Igaku to Seib. (M.a.B.) 1953:*29*:59.
21. Yoshikawa, H., and Ishimaru, S.: Science (Japan) 1943:*13*:62.

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AMYLOIDOSIS AS A STRESS DISEASE

by

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Some of our earlier work induced the hypothesis that amyloid-osis is to be included among the diseases of the stress syndrome. It has been noted in experimental and clinical amyloidosis the presence of neutrophilia, monocytosis and lymphopenia, changes in the blood that point to the stress syndrome (4, 7). In further experiments degeneration and atrophy of the lymphoid tissue of spleen was observed (14) as has also been demonstrated that corti-sone promotes amyloid deposition (3, 4, 11). Furthermore, amyloido-sis is promoted by castration and to some extent also by thyroid-ectomy (4).

The purpose of the investigation now to be described was to study the validity of our theory of the association of amyloidosis with stress. In view of this we assessed the weights of some glands of certain animals in which amyloidosis was produced by means of caseinate, and certain histo-pathological changes. Since, moreover, stress retards growth and in some cases leads to hypofunction of the thyroid and gonads, we have attempted to counteract amy-loidosis with somatotropin, thyreotropin and testosterone. Notice has also been given to the share of infection in the development of caseinate amyloidosis.

MATERIAL AND METHODS

The experimental animals were adult male white mice. Before the series of tests were started, the animals were kept for three weeks on a diet of oats and water only, as it is more difficult to induce amyloidosis on the ordinary full diet. The test lasted 45 days in all groups, after which the animals were killed by decapitation. The animals were weighed at the beginning and end of the test, and after decapitation the liver, spleen, kidneys, thymus, hypophysis, thyroid, adrenals and testicles were weighed. The endocrine glands and liver were fixed in formalin; in addition pieces of spleen were fixed in Zenker's fluid and lead acetate and pieces of liver in the manner of Gendre. The following stainings were used: hematoxylin-congo red according to Highman and toluidin blue for amyloidosis studies, Sudan IV as a fat stain and Best's carmine for glycogen, and hematoxylin eosin as a general stain. The percentage of amyloidosis was determined by using the line sampling method of Uotila and Kannas (13). Not less than 10 samples were obtained from each spleen and one visual field was measured on each sample. Thus the amyloid percentages are means of 10 readings. Macroscopically infected animals were discarded from the final results.

RESULTS

Four groups of mice were treated as follows:

1) *Controls:* 15 animals, which were given subcutaneously 0.5 ml of physiological saline every day for 45 days. In the course of the test 3 mice died and 4 became infected. The final group thus consisted of 8 animals.

2) *Caseinate group:* 15 animals were given subcutaneously 0.5 ml of 5% sodium caseinate solution on 45 days. During the test 1 animal died and 1 became infected. 13 animals remained to make up the final group.

3) *Caseinate-somatotropin-thyrotropin group:* 15 animals received daily 0.5 ml of 5% caseinate, 1 tibia unit of somatotropin (Somacton)[1] in 0.2 ml physiological saline acidified by HCl, and 0.05 USP unit of thyrotropin (Actyron)[1] in 0.2 ml of physiological saline. During the experiment three animals died and 4 became infected so that the final group counted 8 animals.

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[1] One tibia unit is the minimum effective dose in the tibia test according to Greenspan *et al.*, Endocrinology 45, 455, 1949.

Actyron and Somacton we received through the courtesy of Dr. Fredirik Paulsen, M. D., Nordiska Hormonlaboratoriet Ab., Malmö, Sweden.

4) *Caseinate-somatropin-thyrotropin-testosterone group:* 25 mice were treated as in group 3, but in addition they received 10 mg of testosterone (Perandren M)[1] on the first and 21st day of injections. From this group 8 animals died during the experiment and 5 animals were infected. The final group consisted of 12 animals.

The effect of different treatments on the weights of the animals and their organs as well as on the amyloidosis percentage in the spleen are shown in Table 1.

a) *The Effect of Caseinate Injections*

A comparison made between the control and caseinate groups shows that among the animals kept on a diet and which were given physiological saline only one out of eight (12.5%) developed amyloidosis without macroscopically demonstrable infection. In the caseinate group the percentage of infection of amyloidotic mice was 77. The amounts of amyloid in the control and caseinate groups exhibit a statistically significant difference (P = 0.002). Pyroninophilia in the lymphoid and reticuloendothelial tissues of the spleen seems to be somewhat further advanced in the controls than in the caseinate animals. In amyloidotic areas this development is always poor (Fig. 4). The weight of the spleen is distinctly lower in the caseinate group than in the controls.

Thyroid. — In the thyroids of the controls the cells were mainly cubiform and presented a hyperactive appearance; the follicles were deficient in colloid. In the caseinate group the thyroids are richer in colloid and the cells flatter. In comparison with the controls, the function had decreased (cf. Figs. 1 and 2).

Liver. — Both the controls and the caseinate animals fairly regularly exhibit small colonies of inflammatory cells. Glycogen was established in two controls only. Fatty degeneration of hepatic cells is clearly more common and more intense in the caseinate group (cf. fig. 3). The weight of the liver is equal in both groups.

Thymus. — Although the organ was weighed, the fat could not be separated with sufficient accuracy to give validity to the weights. Histologically 54% of the animals in the caseinate group showed clear atrophy of the thymus, but among the controls only one.

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[1] The Perandren used was obtained through the courtesy from J. A. Tamminen, M. A., the representative of »Ciba», Manufacturing Chemists.

TABLE

TABLE SHOWING THE RESULTS

Treatment	Group no.	Number of Mice	Weight at the Beginning and End of the Test and Difference in Grams
Physiological saline	1	8	30.4 28.8 — 1.6 g
Sodium caseinate	2	13	30.4 29.2 — 0.8 g
Sodium caseinate + 1 unit STH + 0.05 USP TSH ......:..............	3	8	29.8 27.0 — 2.8 g
Sodium caseinate + 1 unit STH + 0.05 USP TSH + 2 times 10 mg Testo-sterone	4	12	29.2 27.4 — 1.8 g

Adrenals. — In the caseinate group the weight tended to exceed that of the controls. Visually no clear difference was present in lipid content. In the caseinate group, on the other hand, nearly half of the animals exhibited smallish areas of necrosis and haemorrhage.

Testicles. — Although the testicles weigh less in the caseinate group, no difference is revealed histologically.

Hypophysis. — There is no clear difference in weight or cytology.

From the caseinate tests it may be concluded that injections of caseinate produce clear amyloidosis of the spleen. The adrenals are larger than normal and rich in lipids; necrosis and haemorrhage often occur. The spleen, with lymphoid tissue as an exponent, loses weight, the follicles are atrophied The thymus likewise is atrophic. The activity of the thyroid diminishes, as does the weight of the testicles. Lipid degeneration is seen in the liver.

b) *The Effect of Hormone Treatment*

Estimating the above results and those obtained in earlier work it was logically understandable that we should attempt to compensate the endocrine changes produced by caseinate stress by

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IN EXPERIMENTAL STUDIES

Average Weight in mg							Percentage of Amyloid in Spleen
Liver	Kidney	Spleen	Hypo-physis	Thyroid	Adrenals	Testicles	
1400	402.6	132.9	2.0	10.8	6.8	189.3	3.0 ± 3.0
1400	412.9	83.2	1.8	9.4	7.3	174.5	26.0 ± 6.0
1250	403.8	92.9	1.8	8.7	5.6	170.0	44.0 ± 7.0
1290	535.3	75.3	1.6	12.4	7.6	124.0	17.0 ± 4.0

administration of growth hormone, thyrotropin and testosteron to prevent caseinate amyloidosis.

Table 1 shows that under the conditions described thyrotropin and growth hormone treatment did not put a check to but actually promoted caseinate amyloidosis. On the other hand combined thyrotropin-growth hormone-testosterone treatment caused a reduction in the amyloid percentage. The difference between groups 3 and 4 is thus statistically significant (P = 0.01), likewise that between groups 1 and 4 (P = 0.01), so that the control level was not reached.

From this experiment the conclusion may be drawn that when used to prevent stress, thyrotropin-growth hormone-testosteron therapy seems to reduce amyloidosis.

c) *The Effect of Infection in Amyloidosis Studies*

As stated in the foregoing, the cases exhibiting infection macroscopically were discarded from the final series.

In *the control group* 4 infected animals sui vived to the end of the test. In these the mean percentage of amyloidosis in the spleen was 28. Compared with the value obtained for the non-infected controls, 3%, the percentage is distinctly higher.
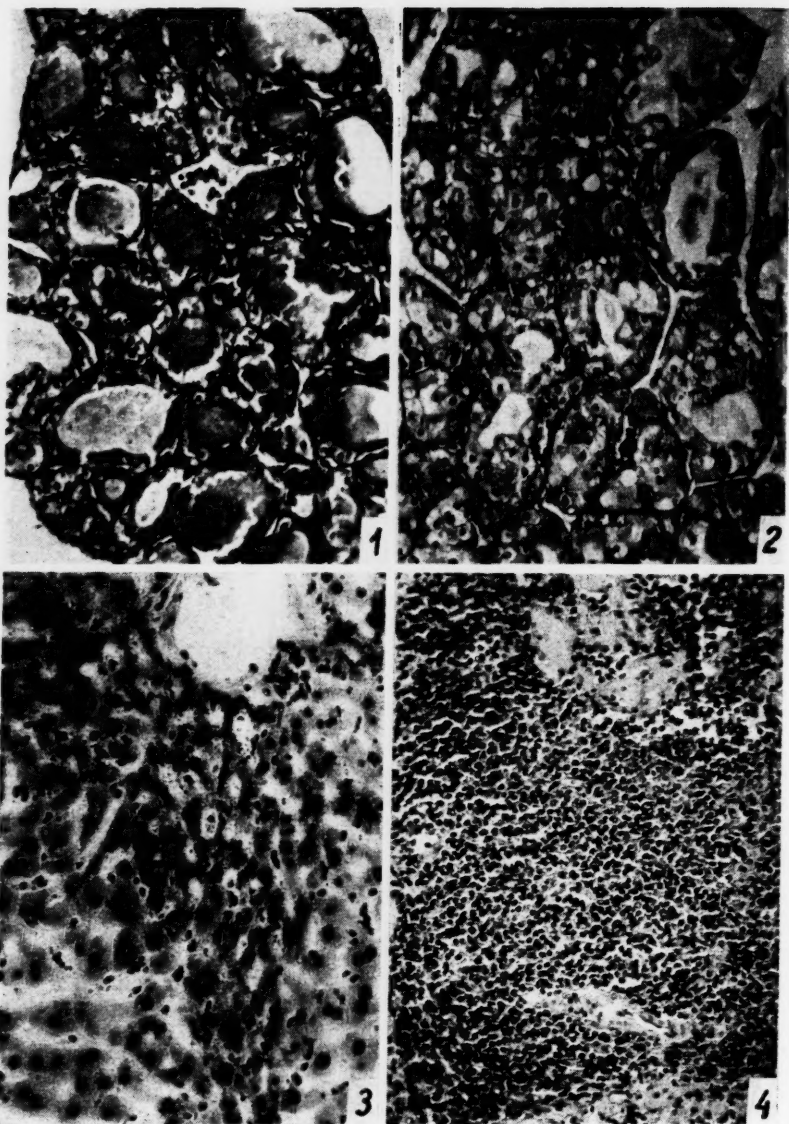
Fig. 1. — Thyroid of a caseinate mouse. A greater amount of colloid and flatter cells than in the controls. Formalin. Koneff stain. 230 ×.

Fig. 2. — Thyroid of a control mouse. Cubiform and cylindriform cells, sparse and poorly staining colloid. Relatively intense hyperfunction. Formalin, Koneff stain. 230 ×.

Fig. 3. — Liver of caseinate mouse. Fatty degeneration of hepatic cells and inflammatory cell infiltrations. Frozen section + Sudan IV stain. 240 ×.

Fig. 4. — Spleen of a caseinate mouse. Two amyloidotic areas and moderately intense pyroninophilia in the area of 11, 13 and 17 hrs, elsewhere slighter. Zenker, methyl green — pyronine stain. 240 ×.

In *the caseinate group* only one infected animal lived to the termination of the test exhibiting in its spleen the amyloid percentage 28, which does not differ from the corresponding figure obtained for non-infected caseinate animals, 26%.

In *the growth hormone-thyrotropin group* 4 infected animals showed an average amyloid amount of 55% and non-infected animals 44% of amyloid. Here, too, infection tended to increase amyloidosis.

In *the growth hormone-thyrotropin-testosterone group* the corresponding figures were 22 and 17%, which bore witness to the same state of things.

From the foregoing it appeared obvious to us that an infection noted macroscopically may in itself produce amyloid and may accelerate caseinate amyloidosis.

DISCUSSION

The described findings show clearly that in caseinate amyloidosis we are concerned with stress changes. In view of what is known of caseinate amyloidosis there is no doubt that the stressors in question are caseinate injections, the antigen-antibody reactions induced by them, and multiple dietary deficiencies.

An analysis of the organic changes caused by caseinate injections showed that the adrenals were larger than normal and rich in lipids as in the adaptation phase of stress syndrome. The weight of the spleen diminished and its lymphoid tissue as well as that of the thymus became atrophied as they usually do in stress. The activity of the thyroid, and the weight of the testicles, characteristically, diminished, and lipid degeneration was present in the liver. When these findings are supplemented by the neutrophilia, monocytosis and lymphopenia which Peräsalo *et al.* and Latvalahti noted we believe we are justified in speaking of caseinate amyloidosis as a stress disease.

Our results also seem to suggest that secondary infection causes acceleration of amyloidosis, which is understandable from what was stated above. Another factor promoting amyloidosis is ascorbin acid deficiency. Since cortisone and for instance heat stress (Peräsalo *et al.*) accelerate amyloidosis, obviously factors promoting stress are on the whole promotive of amyloid deposition. Yet

among these ascorbin acid deficiency — as a factor which impairs
the mesenchyma — seems to be particularly important (5, 6, 9, 10).

We have previously brought forward evidence in support of the
association of the pathogenesis of amyloidosis with the RES,
regardless of whether this tissue is in the spleen, liver or kidney.
The onset of amyloidosis is preceded in the spleen by hypertrophy
of the rediculoendothelial tissue and is followed by its atrophy
(Uotila *et al.*). In the present study we noted that the cells in
amyloidotic areas did not stain with pyronin and thus were poor
in ribonucleic acid. In contrast to this the reticular cells between
the amyloidotic areas stained fairly well with pyronin. This seems
to us to speak for the possibility that in amyloidosis the reticulo-
endothelial tissue has become activated to such an extent that
especially around the spleen follicles it degenerates and gets sur-
rounded with amyloid.

If amyloidosis is granted the character of stress, it follows that
therapy which aims at compensating the minus components of
stress by increasing the growth hormone and intensifying the
function of the thyroid and gonads causes remission of amyloidosis.
This, we presume, accounts for the anti-amyloidotic effect of
combined somatotropin-thyreotropin-testosterone therapy.

It struck us that in earlier studies we obtained by combined
somatotropin-thyreotropin treatment an anti-amyloidotic effect,
but in present experiments this treatment furthered amyloidosis.
The explanation apparently must be sought in the fact that the
preparation 'Actyron' was in the past standardized in Heyl-
Laqueur units, at present in USP units. Not aware of the actual
ratio of these units when planning the experiments, we gave the
present mice a 2—3 times larger dose of thyrotropin than the earlier
ones. Since thyrotropin accelerates amyloidosis (14), we obviously
have had to do with a relative excess of thyrotropin over somato-
tropin. The reciprocal ratio of somatotropin and thyrotropin may
therefore be a factor worth taking into account in experiments of
this kind.

It is always hazardous to adapt conclusions from animal experi-
ments to human patho-physiology. Yet, as a hypothesis to prompt
further work, we should to like to suggest the following: The pre-
vention or curing of amyloidosis apparently necessitates that the
primary and specific factor causing amyloidosis be removed. The

consideration nearest in importance is compensation of the stress and its non-specific minus components. Further, media which have a stimulating effect on the whole mesenchyma, such as ascorbic acid, should be made use of.

SUMMARY

The effect of caseinate injections on mice kept on a scorbutogenic diet has been examined. The adrenals were found to increase in size and be rich in lipids. The weight of the spleen diminishes and its lymphoid tissue as well as that of the thymus become atrophied. The activity of the thyroid declines, the testicles lose weight and fatty degeneration is noted in the liver. In the authors' opinion the findings seem to prove that in caseinate amyloidosis we are concerned with a stress disease.

Secondary infection accelerates caseinate amyloidosis. Also without caseinate may it produce amyloidosis.

Combined thyrotropin-somatotropin treatment, in contrast to earlier findings, accelerated caseinate amyloidosis. The authors account for this by the relatively larger amount of thyrotropin used than in the earlier experiments.

Combined somatotropin-thyrotropin-testosterone treatment produced remission of caseinate amyloidosis.

REFERENCES

1. GREENSPAN, F. S., LI, C. H., SIMPSON, M. E., and EVANS, H. M.: Endocrinology 1949:*45:*455.
2. IVERSEN, K.: In ASBOE-HANSEN, G.: Connective Tissue in Health and Disease. — Munksgaard, Copenhagen 1954.
3. LATVALAHTI, J.: Acta Path. Scand. 1952:Suppl. *93:*81.
4. LATVALAHTI, J.: Acta endocrinol. 1953:Suppl. 16.
5. LETTERER, E.: Ärztliche Forschung 1949:*3:*274.
6. LETTERER, E.: Wirchows Archiv für Path. Anat. 1949:*1:*317.
7. PERÄSALO, O., KUUSISTO, A., and LATVALAHTI, J.: Ann. Med. Exper. et Biol. Fenn. 1949:suppl. 2.
8. PERÄSALO, O., KUUSISTO, A., and HALONEN, P. I.: Ann. Med. Int. Fenn. 1950:*39:*34.

9. PIRANI, C. L., BLY, C. G., SUTHERLAND, K., CHERESO, F.: Science 1949:*110*:145.
10. SELYE, H.: Stress. Acta Inc., 1950 Montreal.
11. TEILUM, G.: Ann. Rheumat. Dis. 1952:*11*:119.
12. TEILUM, G.: J. Lab. and Clin. Med. 1954:*43*:367.
13. UOTILA, U., KANNAS, O.: Acta endocrinol. 1952:*11*:49.
14. UOTILA, U., PERÄSALO, O., and VAPAAVUORI, M.: Acta Path. Scand. 1955:*37*:322.

FROM THE DEPARTMENT OF FORENSIC MEDICINE, AND THE THIRD
SURGICAL UNIVERSITY CLINIC, HELSINKI

EFFECT OF PARA-AMINOSALICYLIC ACID (PAS) ON THYROID ACTIVITY

AN EXPERIMENTAL HISTO-QUANTITATIVE STUDY

by

O. PERÄSALO and P. TALA

Recent investigations have shown that the drugs used extensively in the treatment of tuberculosis have a definite effect on the function of endocrine glands. The relation between the thyroid gland and tuberculosis has been dealt with in a number of studies. According to experimental studies administration of a tuberculostatic substance, para-aminosalicylic acid (PAS), in therapeutic doses for 10 days produces moderate thyroid hyperplasia in rats. This change is prevented by thyroxine but not by sodium iodine and is not enhanced by methyl thiouracil (5). Wong *et al.* observed a reduced I^{131} uptake but no effect on the pituitary-adrenal axis after 2 weeks' administration of ⁓PAS to rats. Beattie and Chamber (2) noted, for instance, that para-aminosalicylic acid (PAS sodium salt) given to rats under the experimental conditions described produced a fall of radio-iodine uptake to 10% of the control values at the end of 16 days. After 30 days this low level is unchanged.

PAS has also been found to prevent formation of diiodotyrosine and the thyroid hormone (1). This effect falls only slightly short of that of thiouracil. Hanngren (3) showed that in man the rate of uptake of 1^{131} by the thyroid gland was reduced by an intravenous

injection of PAS. According to the studies of Pätiälä and Isotalo (7) para-aminosalicylic acid administered for 4 weeks caused a significant decrease in the percentage of epithelium in the guinea pig thyroid as compared with the control group.

Our purpose was to study experimentally the effect of PAS on the thyroid gland, using as earlier as a criterion of thyroid function the linear measurements of the histo-quantitative method of Uotila and Kannas (11), a quick and serviceable method for establishing thyroid activity. Changes in the weight of the thyroid gland after the treatment were also recorded.

MATERIAL AND METHODS

The experimental animals used were young male guinea pigs, of as accurately equal weight as possible. The animals were kept in similar circumstances of light, temperature, and nutrition. The food consisted of grass, Swedish turnips, and oats.

The tests were made with para-aminosalicylic acid (PAS-Cilag cryst, Cilag Aktiengesellschaft Schaffhausen). One millilitre of the solution (100 mg of PAS) was injected subcutaneously once daily into the animal's back. The animals were given 1.0 ml of physiological saline (0.9%). All the animals were killed by decapitation, the most rapid method and the least harmful to the thyroid gland. Both lobes of the thyroid gland were dissected out carefully, cleaned, weighed on a torsion balance and fixed immediately in Bouin's fluid for 6 hours. After fixation the specimens were treated with rising concentrations of alcohol. The left thyroid lobe was embedded in paraffin, then bisected lengthwise with the microtome into two equal halves, and eight to ten slices were cut at about 3 μ from the median surfaces. Staining was by Koneff's modification (6) of Mallory's Azan methods as used by Tala (8) in earlier experiments. The linear measurements for calculation of the percentage of epithelium in the thyroid gland were performed by the histo-quantitative method devised by Uotila and Kannas (11) and used by Tala (8, 9), Kannas and Tala (4), Tala, Railo and Elfving (10), and Pätiälä and Isotalo (7) for determining the proportion of different components in the thyroid gland. Several sections made from the median planes were placed in an apparatus, microprojector, by which the image was projected at a magnifica-

Fig. 1.

Fig. 2.
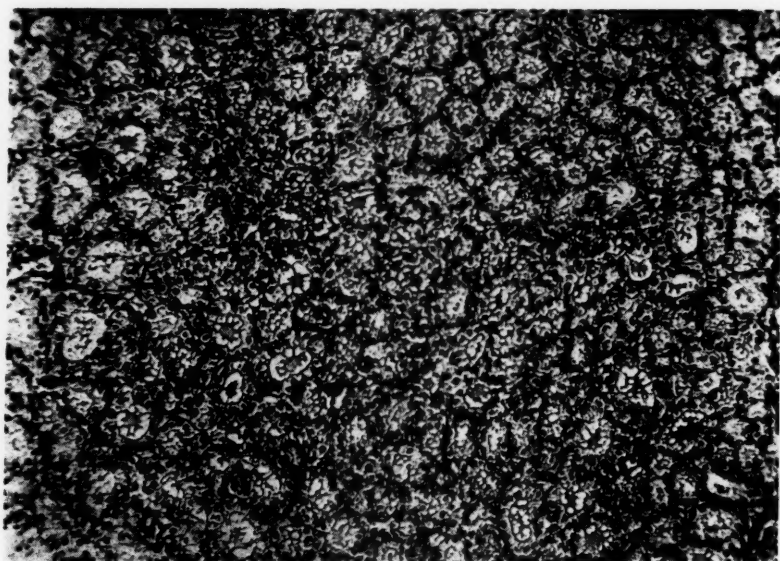
Sections representative of the average percentage of epithelium in guinea-pig thyroids. Koneff's modification of Mallory's Azan stain $\times 135$.

Fig. 1. — Control group (E% = 80), saline alone.

Fig. 2. — Group 3 (E% = 92), after treatment with para-aminosalicylic acid for 18 days.

tion of 400 diameters on a white screen, on which two lines, each 40 cm in length and intersecting to form an X, had been drawn. Measurements in millimetres were then made of the epithelium, colloid and stroma, respectively along the full length of the two lines. The total length of each component in the four sections was examined and was calculated as a percentage of the total length of the lines. The percentage of epithelium (E%) was used as a criterion of thyroid activity, since the proportion of stroma was found to remain fairly constant.

RESULTS

The experimental animals were divided into three groups of 5 guinea-pigs each. Seven animals served as controls (Group 4). The animals in Group 1 were given 100 mg of PAS per day by subcutaneous injection on 6 days and were killed on the 7th day. Those in Group 2 received the same daily dose for 12 days and were killed on the 13th day, and in Group 3 the injections were continued for 18 days, with autopsy on the 19th day.

In the control group, 4 guinea pigs received physiological saline subcutaneously for 6 days, and another 3 for 12 days. They were killed by decapitation on the day following the last injection.

The results obtained are shown in Table 1. It will be noted that with prolongation of the PAS treatment the average epithelial percentage (E%) in the thyroids in each group increased. PAS, accordingly, produced hyperplasia and activation of the thyroids. The difference between Group 3 and the control group is almost significant. On the other hand, no clear change could be noted in the relative weights of the thyroid glands.

DISCUSSION

The experiments support the observation reported by some workers, that PAS produces increased activity of the thyroid gland. This increase was determined objectively in numerical terms by the histo-quantitative method of Uotila and Kannas (11), the accuracy and serviceability of which has been tested by Tala in a number of previous experiments (8; 9), and by Kannas and Tala (4). The relative weight of the thyroid gland, though not — as we showed in a previous study — equally suitable as a criterion of thyroid

TABLE 1

PERCENTAGE OF EPITHELIUM AND WEIGHT OF THYROID GLAND IN MALE GUINEA-PIGS TREATED WITH PAS FOR 6, 12 AND 18 DAYS

Guinea-pig N:o	E %	Body Weight (g)	Thyroid Weight (mg)	$10^5 \cdot \dfrac{\text{Thyr. Wt.}}{\text{Body Wt.}}$
Group 1. Six daily injections of PAS (100 mg/day); animals killed on 7th day				
1	62.7	308	36	11.7
2	76.3	298	34	11.4
3	80.3	364	42	11.5
4	81.7	310	49	15.8
5	77.3	312	37	11.9
Aver. E % 75.7 ± 6.7			Aver. relat. wt. 12.5 ± 2.0	
Group 2. Twelve daily injections of PAS (100 mg/day); animals killed on 13th day				
10	78.0	382	48	12.6
11	76.9	420	47	11.2
12	76.1	352	54	15.4
13	81.3	394	46	11.7
14	87.4	252	27	10.7
Aver. E % 79.9 ± 4.1			Aver. relat. wt. 12.3 ± 1.7	
Group. 3. Eighteen daily injections of PAS (100 mg/day); animals killed on 19th day				
18	80.7	390	60	15.4
19	86.7	268	39	14.6
20	81.7	390	51	13.1
21	96.9	170	25	14.7
22	92.7	216	29	13.4
Aver. E % 87.7 ± 6.3			Aver. relat. wt. 14.2 ± 0.9	
Group 4. Controls, daily injection of 1.0 ml physiological saline into Nos 6—9 for 6 days (killed on 7th day) and Nos. 15—17 for 12 days (killed on 13th day)				
6	74.3	392	53	13.5
7	79.7	334	35	10.5
8	78.5	380	55	14.5
9	85.0	246	35	14.2
15	82.9	250	42	16.8
16	79.5	280	45	16.1
17	77.9	248	35	14.1
Aver. E % 79.7 ± 3.2			Aver. relat. wt. 14.2 ± 2.4	

activity as the histological appearance of the gland, has been taken into account in the present study. No clear changes were noted in the relative thyroid weights in the different groups.

SUMMARY

The effect of para-aminosalicylic acid (PAS) on thyroid activity was studied by giving daily subcutaneous injections of this drug to young male guinea pigs. The thyroid activity was determined by the histo-quantitative method of Uotila and Kannas (11).

PAS produced hyperplasia of the thyroid gland, recorded as an increase of the epithelial percentage of the gland (E%). The difference between the group which had been given 18 daily injections of PAS (100 mg daily) and the control group was almost significant. No clear changes were noted in the weight of the thyroid gland. The experiments seemed to point to a goitrogenic effect of PAS under these experimental conditions.

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REFERENCES

1. Angel, R. W., Mayer, S. W., and Morton, M. E.: Am. Rev. Tub. 1955:71:889.
2. Beattie, J., and Chambers, R. D.: J. Endocrin. 1953:10:65.
3. Hanngren, A.: Lancet 1952:2:117.
4. Kannas, O., and Tala, P.: Ann. med. exper. Fenniae 1953:31:124.
5. Kjerulf-Jensen, K., and Wolffbrandt, G.: 1951:7:376.
6. Koneff, A. A.: Stain. Tech. 1938:13:49.
7. Pätiälä, J., and Isotalo, A.: Ann. med. exper. Fenniae 1955:33:25.
8. Tala, P.: Acta Endocrin. 1952:10:Suppl. 9.
9. Tala, P.: Endocrinology 1953:53:474.
10. Tala, P., Railo, J., and Elfving, G.: J. Endocrin. 1953:10:82.
11. Uotila, U., and Kannas, O.: Acta Endocrin. 1952:11:49.
12. Wong, T., Hognes, J., and Williams, R.: Proc. Soc. Exper. Biol. & Med. 1953:82:598.

DETERMINATIONS OF STREPTOMYCIN IN VARIOUS
PARTS OF THE RESECTED TUBERCULOUS LUNG

by

MARTTI TURUNEN, PEKKA HALONEN and TIMO KOSUNEN

We have previously reported a method for the determination
of penicillin in various parts of the resected tuberculotic lung (5).
As particularly in such chronic diseases as in tuberculosis the
distribution of antibiotics over the affected area is of very great
significance, we have adapted this method to streptomycin, and
present below the results of these investigations.

The 6 patients with pulmonary tuberculosis were males and
females, aged 27 to 39 years and weighing 59 to 74 kg. They had
all received antituberculous drug treatment for some time and had
been admitted to a surgical hospital for pneumonectomy or
lobectomy. One hour and a half before the operation an intramus-
cular injection of $2\frac{1}{2}$ g dihydrostreptomycin was given. During the
operation when exactly 2 hours had elapsed since the drug injection,
a blood specimen was withdrawn for determination of the serum
streptomycin lewel.

The preparation of lung samples was identical with that
described in our study concerning penicillin (5). The principles of
the study referred to were also applied for the evaluation of the
histological tissue samples. For the determination of the dihydro-
streptomycin[1] concentration the samples were weighed and extracted

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[1] The streptomycin determinations were carried out at the Microbiological
Department of Lääketehdas Orion.

TABLE 1
STREPTOMYCIN PER GRAMME OF TISSUE IN TUBERCULOUS PROCESSES

Patient 1			Patient 2			Patient 3		
1. Serum	38	ug	1. Serum	17	ug	1. Serum	34.2	ug
2. Normal lung tissue	52	»	2. Normal lung tissue	2.9	»	2. Normal lung tissue	6.2	»
3. Normal bronchus	27.6	»	3. Normal lung tissue	19	»	3. Normal lung tissue	11.5	»
4. Caseation	14.3	»	4. Normal bronchus	15	»	4. Normal bronchus stam	7.6	»
5. Wall of cavity	11.1	»	5. Normal bronchus	16	»	5. Normal segmental bronchus	16	»
6. Pleura and wall of cavity	10.7	»	6. Tuberculous granulation	6	»	6. Tubercles	11.5	»
7. Fibrosis	35.9	»	7. Tubercles	3.3	»	7. Caseation	7	»
8. Content of cavity	6.1	»	8. Fibrosis	23	»	8. Fibrosis	10.5	»
9. Tuberculous lymph gland	15.6	»	9. Tuberculous lymph gland.	8.7	»	9. Fibrosis	9.6	»

Patient 4			Patient 5			Patient 6		
1. Serum	8.9	ug	1. Serum	7.8	ug	1. Serum	5.7	ug
2. Normal lung tissue	14	»	2. Normal lung tissue	7.9	»	2. Normal lung tissue	4.5	»
3. Normal bronchus	4.3	»	3. Normal bronchus	6.3	»	3. Normal bronchus	7	»
4. Bronchus and peribronchial tbc.	4.5	»	4. Caseation	5.9	»	4. Caseation	6.5	»
5. Caseation	11.5	»	5. Fibrosis	8	»	5. Wall of cavity	6.2	»
6. Caseation	8.4	»	6. Fibrosis	6.9	»	6. Fibrosis	2.9	»
7. Wall of cavity	7.1	»	7. Fibrosis	5.9	»	7. Hypertrophic pleura	2.0	»
8. Hypertrophic pleura	3.1	»	8. Fibrosis	7.4	»	8. Content of cavity	7.1	»
9. Tuberculous lymph gland	5.3	»						

with 1 ml of phosphate buffer solution at pH 8.0. The dihydro-streptomycin assay method used was the »Cylinder-Plate Method Using Bacillus Subtilis as the Test Organism» (2), with minor modifications. The destruction of the effect of penicillin in the samples of patients who had been administered penicillin was carried out with the aid of penicillinase. The dihydrostreptomycin concentrations in various parts of the tuberculotic lung, as well as the level of dihydrostreptomycin in serum and tissue samples, are shown in the table 1. In table 2 the serum/tissue ratio of the streptomycin concentrations are given and grouped according to type or location of sample.

On studying the table 1 it can be noted that very considerable variations occur in the serum dihydrostreptomycin concentrations. In general the serum values lie on a fairly high level, as do also the values of normal lung tissue, bronchial tissue and fibrotic tissue.

TABLE 2

STREPTOMYCIN CONCENTRATIONS IN TUBERCULOUS PROCESS AS THE RATIO BETWEEN THE STREPTOMYCIN CONCENTRATION IN THE SERUM AND IN THE TISSUE

Sample	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Normal lung tissue	0.73	5.85 / 0.9	5.5 / 3	0.64	0.99	1.25
Normal bronchus..	1.4	1.05 / 1.15	4.5 / 2.2	2.1 / 2.0	1.25	0.82
Caseation	2.65		4.9	0.77 / 1.05	1.3	0.82
Wall of cavity	3.45 / 3.5			1.25 / 2.9		2.8
Fibrosis	1.05	0.74	3.6 / 3.3		0.98 / 1.1 / 1.05 / 1.3	1.95
Content of cavity	6.2					0.92 / 0.8
Tuberculous granulation..........		2.85				
Tubercles		5.2	3			
Tuberculous lymph gland	2.4	1.95		1.7		

Abundant amounts of dihydrostreptomycin were also found in the cavity walls. Even inside the cavities and in the caseous necrosis values were detected which fully inhibit the growth of the human tubercle bacillus, providing the bacilli have normal sensitivity. The slightest dihydrostreptomycin concentrations were found in the thickened pleura.

DISCUSSION

It was observed in the course of this study that dihydrostreptomycin, when used in doses of at least $2\frac{1}{2}$ g, rapidly spread to various parts of even such lungs which were affected with severe tuberculosis, in concentrations which may be interpreted as sufficient to inhibit the growth of the human tubercle bacillus of normal sensitivity. Similar conclusions have been reached also by Steenken, D'Esopo and Wolinsky (4), as well as Canetti and Grumbach (1).

In tissues, which can be clearly differentiated under macroscopic examination, as for instance in bronchial tissue and lymph nodes, the relations between serum and tissue dihydrostreptomycin seem to be closest to each other, whereas, if samples are taken from several parts of a lung, it is more difficult to control the homogeneity of the specimens, and the extensive variations which occur in samples histologically similar to each other may be partly explained by this circumstance. On the other hand, it should be also borne in mind that Nelson, Forgacs and Kuceva (4) have noted that streptomycin particularly tends to pass into reticuloendothelial tissue, which is also apt to produce variations within histologically similar tissue samples, especially depending on the duration of the process.

There are only a few mentions in the literature of streptomycin determinations in lung tissue. Our series is also so restricted that it does not allow any definite conclusions, but at least it should appear that we have obtained fairly consistent results by the method employed, even if dispersion has been considerable in some cases. It was of interest to note that rather abundant amounts of streptomycin were found in the contents of the cavities and in the cheesy masses of caseous necrosis.

SUMMARY

The authors have adapted to determinations of streptomycin in various parts of the resected tuberculotic lung a method previously described by them for the determination of penicillin in lungs samples. Streptomycin spread everywhere, even into the cavities, in such abundant amounts that it can be considered to inhibit the growth of the human tubercle bacillus, provided the strain involved is of normal sensitivity. The largest amounts of streptomycin were found in the lung tissue and bronchial walls, the smallest in necrotic tissue and the thickened pleura.

REFERENCES

1. CANZETTI, G., and GRUMBACH, F.: Ann. de L'institut Pasteur 1953: 85:380.
2. GROVE, D. C., and RANDALL, W. A.: Assay Methods of Antibiotica. — Medical Encyclopedia, Inc. New York 1955.
3. NELSON, W. E., FORGACS, J., and KUCESA, J. L.: Proc. Soc. Exp. Biol. and Med., 1947:64:20.
4. STEENKEN, W. JR., D'ESOPO, N. D., and WOLINSKY, E.: Am. Rev. Tuberc. 1947:56:403.
5. TURUNEN, M., and HALONEN, P.: Ann. Med. Exper. et Biol. Fenniae. 1956:34:383.

THE ABO BLOOD GROUP AND CARCINOMA OF THE STOMACH

by

MARTTI TURUNEN and MIKKO PASILA

Recent years have seen awakening interest in the study of genetic factors in disease, particularly in investigations bearing on the genesis of malignant growths. Determinations of blood groups have been regarded as a natural genetic consideration. Yet it has not proved possible to demonstrate to any considerable extent the relation between certain blood groups and various diseases. D. Struthers (1951) found in Glasgow that the occurrence of the A blood-group was more frequent among children who had succumbed to bronchopneumonia than among the general population. The observation of McConnell, Pyke and Roberts (1956) that the A group occurred in a significant majority of patients with diabetes mellitus was also of interest. Aired et al. (1953) found in a fairly extensive series that the A blood-group was markedly more frequent in patients affected with ventricular carcinoma. The increase of group A took place at the expense of group O, whereas blood-groups B and AB remained unchanged. Similar conclusions were reached by Koster et al. (1955). Jennings et al. (1956) published a small series, in which they made comparisons between the blood group distribution of growths situated in different parts of the stomach, as well as between the two sexes. They found that cancer localised to the pyloric part of the stomach was more frequent in men than in women. This was more or less consistent with those percentages which have been reported on the increase of the A blood-group in carcinoma of the stomach. Aird et al. (1954) found that the O-group was more general among

patients with gastric ulcer. It has been also shown statistically
that in patients affected with pernicious anemia the occurrence
of the A group has increased approximately to the same extent
as in ventricular carcinoma. We propose to report a Finnish material
showing the distribution of blood groups among patients suffering
from carcinoma of the stomach.

MATERIAL

The series was collected at the former First and present Second
Surgical Clinic, at the Surgical Department of the Maria Hospital
in Helsinki and at the Southern-Saimaa District Hospital of
Lappeenranta.[1] The series comprised a total of 990 patients with
carcinoma of the stomach, both from urban and rural districts.
There were 613 men and 377 women. All had been ascertained by
biopsies taken at operation. The localisation of carcinoma in the
ventricles was determined with the aid of roentgenological and
surgical findings. On account of the size of the tumour it was not
possible to decide with certainty in 85 cases whether the growth
should be interpreted as belonging to the antrum or the corpus.
A carcinoma of the lung material (440) of the former First Surgical
Clinic of the University of Helsinki was used as the control series.
Determination of the blood groups was carried out at the Depart-
ment of Serology and Bacteriology of the University, Helsinki,
and at the Laboratory of the Southern-Saimaa District Hospital.
The material was collected during a period of 10 years (1946—1956).

The results were treated mathematically by using the x^2-test
to determine whether the distributions of figures x_1, x_2, x_3 and
x_1', x_2', x_3' were mutually similar or dissimilar.[2]

RESULTS

We have divided the series according to the sex distribution
and to the localisation of carcinoma in various parts of the stomach,
and have studied the distribution of blood groups among these
categories. The results are presented in the following Table.

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[1] Our thanks are due to the directors of these clinics, Professor V. Seiro,
Professor R. Tuovinen and Professor E. Tiitinen, for having placed this mate-
rial at our disposal.

[2] We are greatly indebted to Professor Olli Lokki for the mathematical
treatment of the results.

MARTTI TURUNEN AND MIKKO PASILA

TABLE

RELATION BETWEEN VENTRICULAR CARCINOMA AND BLOOD GROUPS

Blood Groups	Pyloric Part	Cardiac Part	Localisation Could not be Determined	Total of All Ventricular Carcinomas
Men				
A	176 = 48.5 % +6.6 %	88 = 43.1 % +1.2 %	19 = 41.0 % —2.0 %	283 = 46.2 % +4.3 %
O	92 = 25.4 % —7.1 %	65 = 31.9 % —0.6 %	16 = 35.0 % +2.0 %	173 = 28.2 % —4.3 %
B	62 = 17.1 % —1.1 %	36 = 17.7 % —0.5 %	10 = 22.0 % +4.0 %	108 = 17.6 % —0.6 %
AB	33 = 9.1 % +1.7 %	15 = 7.4 % ±0.0 %	1 = 2.0 % —5.0 %	49 = 8.0 % +0.6 %
Women				
A	106 = 44.9 % +3.0 %	44 = 43.1 % +1.2 %	19 = 49.0 % +7.0 %	169 = 44.8 % +2.9 %
O	75 = 31.8 % —0.7 %	36 = 35.3 % +2.8 %	9 = 23.0 % —10.0 %	120 = 31.8 % —0.7 %
B	32 = 13.6 % —4.6 %	19 = 18.6 % +0.4 %	6 = 15.0 % —3.0 %	57 = 15.1 % —3.1 %
AB	23 = 9.7 % +2.3 %	3 = 2.9 % —4.5 %	5 = 13.0 % +6.0 %	31 = 8.2 % +0.8 %
Women and Men				
A	282 = 47.1 % +5.3 %	132 = 43.2 % +1.3 %	38 = 45.0 % +3.0 %	452 = 45.7 % +3.8 %
O	167 = 27.9 % —4.6 %	101 = 33.0 % +0.5 %	25 = 29.0 % —4.0 %	293 = 29.6 % —2.9 %
B	94 = 15.7 % —2.5 %	55 = 18.0 % —0.2 %	16 = 19.0 % +1.0 %	165 = 16.7 % —1.5 %
AB	56 = 9.3 % +1.9 %	18 = 5.9 % —1.5 %	6 = 7.0 % ±0.0 %	80 = 8.1 % +0.7 %

Of the 990 cases of ventricular carcinoma under discussion 613 were male and 377 female. Of the carcinomas 599 were localised to the pyloric part (363 in men and 236 in women), and 306 to the cardiac part (204 in men and 102 in women) and in 85 cases the starting point of carcinoma in the ventricle could not be

determined (46 men and 39 women). According to Nevalinna's statistics, the A blood-group occurs in Finland to the extent of 41.9%, O 32.5%, B 18.2% and AB 7.4%.

It emerged in the course of the study that among male patients affected with carcinoma of the pyloric part the A blood-group was 6.6% more frequent than in the general population in Finland, and reciprocally the O blood-group was 7.1% less. In women with pyloric carcinoma the blood-group A was 3.0% more and O group 0.7% less frequent than in the normal distribution of these blood-groups. In pyloric carcinoma, counting men and women together, the increase of the A blood-group was 5.3% and the decrease of the O blood-group was 4.6%. In pyloric carcinoma the distribution of blood-groups in men and women together differed with a certainty of 95% from the normal distribution of blood-groups in Finland. The difference is still more emphasised if the total of the A group is compared to other blood groups. A certainty of 98% is then obtained.

In carcinoma of the corpus a rise of 1.3% was observed in the A group and a rise 0.5% in the O group. In the remaining blood-groups the increase and fall had an average extent of 1%. These differences are not significant statistically.

Using the same mathematical method, a study was made of Aird's and Holländer's series. In both the blood-group distribution in pyloric carcinoma was, with a certainty of 99.9%, different as compared to the normal distribution of blood groups.

In histologically ascertained cases of pulmonary carcinoma (440) which were used by us as controls the blood-group distribution did not differ from that of the general population in this country.

DISCUSSION

According to the literature and in this series as well, patients affected with carcinoma of the pyloric part seemed to belong to the A blood-group to a higher extent than might be expected on the basis of the normal blood-group distribution of the population. However, this phenomen was not as clear-cut in the series now reported as in those previously published, but nevertheless, it was significant statistically (98%). A more frequent occurrence of the

A group in patients with pyloric carcinoma took place in all series mainly at the expense of the O group.

When such statistics of carcinoma in different parts of the stomach are compiled, different possibilities of error are abundant. Attempts were made to reduce the errors as much as possible by gathering material from different hospitals in which both the rural and the urban population are treated. Moreover, only such cases have been included into the series in which the localisation of the growth could also be established at operation and its nature simultaneously ascertained by histological studies.

Carcinoma of the pyloric part of the stomach is more frequent in men than in women (according to the literature 1.9: 1). In addition, the statistics also show that of carcinomas of the stomach 50—80% occur in the antrum. The same proportion was established in the present series also. It is interesting that both antrum carcinoma and pernicious anemia are centralised to the pyloric part, conditions, in which according to the literature the ratio of the A blood-group has increased. It would then be natural to expect that some common biological factor might be the cause, considering that it has also been shown that a person suffering from pernicious anemia is more liable than normal to the onset of carsinoma of the stomach. Both pernicious anemia and pyloric carsinoma are linked with the anatomy and physiology of glands in this region (secretin, intrinsic factor, cobalt metabolism. — On the other hand, no correlation could yet be established between the A group and this phenomenon. It would appear most probable to suppose that in hereditary factors the A blood-group and some anatomic-physiological characteristic of the antrum region run parallel to each other. The occurrence of A can hardly be interpreted as solely a degenerative phenomenon, since an increase of this group could not be demonstrated in other forms of carcinoma.

SUMMARY

The series comprised 990 patients with carcinoma of the stomach, histologically ascertained at operation, from four different hospitals in Finland. The patients belonged to both the urban and the rural population. The male ratio was higher, which is consistent with the literature. Likewise carcinoma was more

frequent in the pyloric than in the cardiac part. The A blood-group occurred with higher frequency in antrum carcinoma than what might be expected on the basis of the normal blood-group distribution of this country (with 98% certainty). This rise occurred at the expense of the O group. In series previously reported in the literature the proportion of A in pyloric carcinoma was higher than in this study. Since the A blood-group is more frequent also in pernicious anemia patients than in the whole population, the authors assume the existence of some genetic correlation between the blood-groups and the anatomy and physiology of the antrum.

REFERENCES

1. Struthers, D.: Brit. J. soc. Med. 1951:5:223.
2. Mc Connell, R B., Pyke, D. A., and Roberts, J. A. F.: British Med. Journ. 1956:1:772.
3. Aird, J., Bentall, H. H., and Roberts, J. A. F.: British Med. Journ. 1953:1:799.
4. Køster, K. H., Sindrup, Erik, and Seele, Vagn.: Lancet 1955:2:52.
5. Jennings, Denys, Balme, R. H., and Richardson, J. E.: Lancet 1956:2:11.

EFFECTS OF ANESTHESIA AND OPERATION ON BLOOD SUGAR

AN EXPERIMENTAL STUDY ON NORMAL AND ALLOXANDIABETIC RABBITS

by

MARTTI TURUNEN and MIKKO PASILA

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Surgery as such is always a strain on the organism. The larger the operation, the greater are the postoperative disturbances in the hormonal, fluid, ionic and other balances; the greater is also the danger of causing irreparable changes. If a hormonal disturbance, which is only artificially maintained at equilibrium, exsists already prior to operation, the dangers associated with operation will be even more considerable. The most common hormonal upset the surgeon is faced with is diabetes mellitus. Formerly the dose of insulin was doubled on the day of operation and after it care was taken to supply extra insulin. This was necessary, because the anesthetic employed was almost invariably ether. Ether is known to be a substance which elevates blood sugar. This has been studied *e.g.* in connection with the glucose tolerance test, and it was noted that in normal individuals ether brings about within half an hour a rise of up to 200 mg% in blood sugar. In two hours it returns again to normal (1). In diabetics the rise is equally abrupt, but the return to normal values takes several hours.

Diabetes as produced experimentally in animals by alloxan is serviceable for studies. According to investigations, the hyperglycemia stage in alloxan diabetes is induced by destruction of beta-cells in Langerhans' islands. Alloxan is a poison which simul-

taneously damages the liver and the kidneys as well, if administered in such quantities as are necessary to produce diabetes in test animals (2). It is noteworthy that similar diabetes as the one brought about in experimental animals by alloxan, can also be induced by dehydro-ascorbic acid, which is a constituent of the normal organism. Glutathione and cysteine, on the other hand, act as protective substances against both dehydro-ascorbic acid and alloxan. In other words, diabetes does not appear, if *e.g.* cysteine is injected simultaneously with alloxan. The idea has also been put forward that alloxan is formed in the organism, and that it plays a possible rôle in human diabetes (3).

The concentration and velocity of injection of the alloxan solution are in direct correlation with the certainty of inducing diabetes in test animals. The greater the rapidity in carrying out the injection and the stronger the solution, the more certain is the genesis of diabetes, but also the higher the mortality of the animals. One can regard as optimal value 100 mg/kg in an intravenous injection of 10 minutes' duration. The mortality rate is about 40 per cent, and the certainty of producing diabetes also 40 per cent. This dosage has been employed by us in our investigations.

The purpose of this study was to examine the effect of the substances generally used in preoperative preparations, *i.e.* chlorpromazin[1] and N-(2'-Dimenthylamino-propyl)- phenothiazin hcl.[2] on the blood sugar level of alloxan-diabetic rabbits. Largactil has recently become a subject of interest in investigations. Its effect on heightening the potency of analgetics and sedatives has been generally recognised. It has been found that it produces an elevation of blood sugar when used in therapeutic doses. It probably cuts off the neurogenic routes between the hypophysis and hypothalamus, thus producing a neurohumoral effect.

MATERIAL AND METHODS

The series consisted of 54 healthy adult rabbits, in 44 of which alloxan-diabetes was induced by injections of »Merck's Alloxan puriss» 100 mg/kg. The time of injection was 8—10 min. Before and after the injection, as well as in connection with certain pro-

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[1] »Largactil» May & Baker.
[2] »Atosil» Bayer.

ceedings, the blood sugar was studied by the method of Creselius-Seyfert. The material was grouped in such a manner that the 10 test animals, in which no diabetes had been induced, formed the control series. In 3 of them the effect of urethane and in 7 the effect of the Atosil-Largactil combination on the blood sugar was studied. Similar tests were performed on the 12 rabbits with alloxan-diabetes; in 6 of them the effect of the urethane injection alone, and in 6 the effect of the Atosil-Largactil injection was examined.

After this observations were made in connection with operations on the influence of the Largactil-Atosil premedication on the blood sugar level. Operations were performed on a total of 16 rabbits, in none of which the diabetes had been counteracted with insulin. The rabbits were anesthetised with 10 per cent urethane, and laparotomy was the surgery recurred to. The test animals were made to fast for 24 hours prior to the operation and postoperatively they were kept on the usual diet. Blood specimens for the determination of the sugar level were taken immediately before the operation or injection from the ear vein, and thereafter every hour for 6 hours, then after 12 hours, and finally samples were withdrawn daily for 3 days, in some cases even for 9 days.

RESULTS

Prior to the alloxan injection the blood sugar of the rabbits was 120—146 mg%. After the alloxan injection 18 test animals died. The cause of death in all rabbits was the hypoglycemic shock following the alloxan injection in about 8—12 hours after it had been given. Shock was proved by blood sugar specimens and on the basis of clinical symptoms. Seven rabbits developed diabetes with a blood sugar varying between 300 and 400 mg%, and 19 rabbits had diabetes with blood sugar variations between 150 and 300 mg%.

Control Series. — Ten rabbits, including 3 healthy animals, were injected with 10 per cent urethane in such amounts that the rabbits went into the analgetic stage, for which 15 to 25 cc of uretan were needed, according to the weight of the animal. The curves on Fig. 1 show the blood sugar values in this group.

As illustrated by the curves, the blood sugar rose by 50—70 mg%. It reached its peak within 2 hours and returned to normal in the course of 2 days.
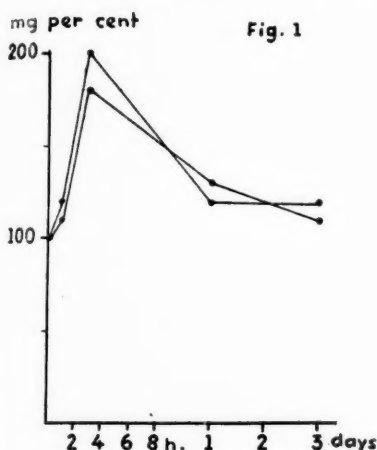
Fig. 1. — Effect of urethane anesthesia on the blood sugar of healthy rabbits.
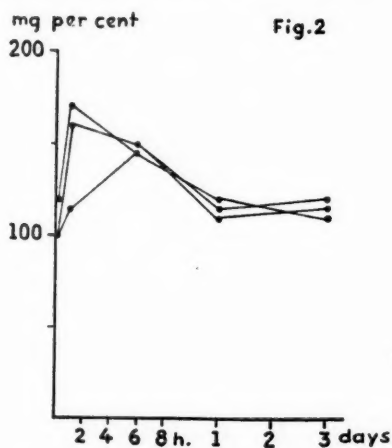
Fig. 2. — Simultaneous effect of chlorpromazin and atosil on the blood sugar of healthy rabbits.

Seven healthy rabbits were injected with 25 mg of Atosil and 25 mg of Largactil, and the specimens were withdrawn as in the foregoing group.

As we note in Fig. 2, the blood sugar concentration of all test animals rose within an hour to its maximum value, which was 70 mg% higher than the initial level. The sugar content returned to its initial value in the course of 1—2 days.

These control experiments show that the effect of urethane on blood sugar on one hand and of the Largactil + Atosil combination on the other was similar, elevating the values within approximately the same time to an approximately similar level.

The effect of urethane on rabbits with alloxan diabetes which had not been counteracted by insulin, was studied in 6 experimental animals. Urethane was given until the analgetic stage was reached (15 to 25 ccm 10 per cent urethane). The results are shown in Fig. 3.

It will be noted that the urethane injection elevates blood sugar by 20 mg%. The peak is reached in 1 hour, after which the curve begins to fall, but has not yet reached its initial value as late as on the third day. The test animals were given no insulin at any stage of the proceedings.

Six diabetic rabbits were administered 25 mg of Largactil and
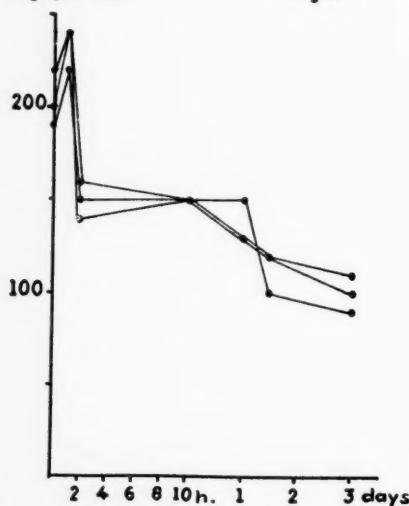
Fig. 3. — The effect of urethane anes-
thesia on the blood sugar of alloxan-
diabetic rabbits.
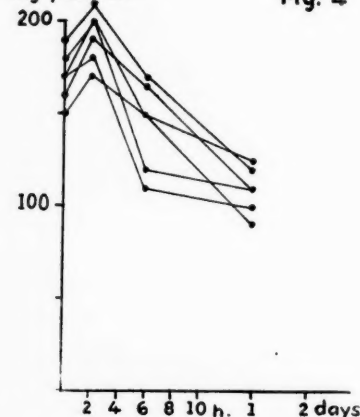
Fig. 4. — Simultaneous effect of
chlorpromazin and atosil on the blood
sugar of alloxandiabetic rabbits.

25 mg of Atosil. The sugar values of these rabbits were determined
as before and the results are shown in Fig. 4.

It will be seen on studying these curves that an elevation by
20 to 30 mg% follows the injection after the lapse of 2 hours.
The blood sugar then falls within 6 hours to a level below the
initial value. After the lapse of 24 hours the blood sugar concen-
tration has not yet returned to the initial level. If a comparison is
made between diabetic and non-diabetic test animals in regard to
the effect of urethane on one hand and of the Largactil-Atosil
combination on the other, it will be noted that in healthy animals
the substances induced a rise of blood sugar of 6—7 hours' duration,
with a peak value exceeding the initial value by 50 to 70 mg%.
In diabetic rabbits under similar conditions a slight rise of about
20—30 mg% was observed during the first two hours, whereafter
there was a fall to below pre-experimental values which persisted
for 1—3 days.

Surgical Series. — The 18 operated test animals fell into the
groups of mild and severe diabetes. For premedication the Largactil-
Atosil combination was used, as in the foregoing experiments. The
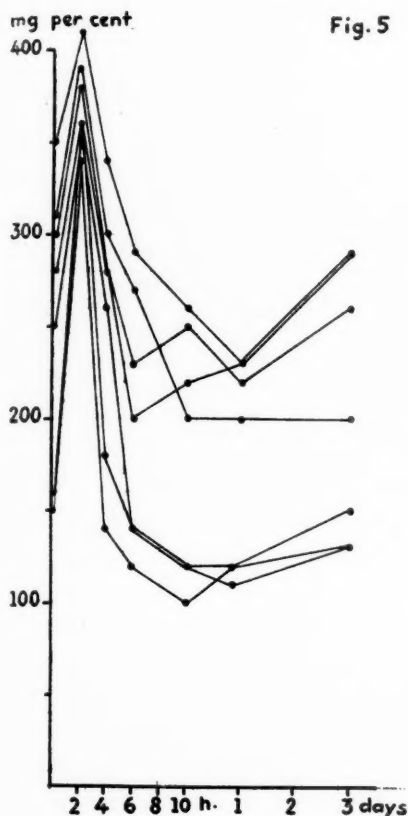
Fig. 5. — The effect of chlorpromazin, atosil, urethane and laparotomy on the blood sugar of alloxandiabetic rabbits.
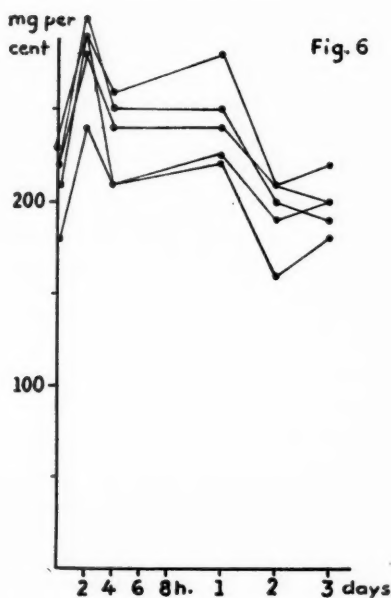
Fig. 6. — The effect of urethane anesthesia and of laparotomy on the blood sugar of alloxandiabetic rabbits.

anesthetic used was urethane, the operation laparotomy. Pre-operative medication was administered 45 minutes before anesthesia. The amount of urethane employed varied between 15 and 25 ccms. The results are seen in Fig. 5.

A considerable rise of the blood sugar level was seen post-operatively in all degrees of diabetes. After the lapse of two hours the sugar concentration of the blood specimen was seen to have reached its peak, and it remained elevated in severe cases for about 24 hours, whereas the fall was more rapid in mild diabetes. In mild cases the rise was about 200 mg%, in cases of moderate severity about 120 mg% and in severe diabetes about 50 mg%,

which means that the values rose to approximately similar levels in all cases. After that the blood sugar returned to normal or even fell below normal in several cases.

Finally 5 diabetic rabbits were examined for blood sugar changes produced by anesthesia and operation without preoperative medication by Largactil and Atosil. These rabbits had diabetes of moderate severity, and they were anesthetised with urethane for the duration of laparotomy. The changes are illustrated in Fig. 6.

As shown in the Figure, there was an elevation of 60 to 80 mg% in the blood sugar. It returned to its original level in the course of 2 days and thereafter persisted on it. Consequently there is no later fall.

<div align="center">DISCUSSION</div>

It was noted in the course of the studies performed on healthy rabbits that urethane used as anesthetic as well as Largactil (M & B) and Atosil (Bayer) used for preanesthetic medication produced an elevation of blood sugar by 70—80 mg%, which persisted for approximately 2 days. According to literature, we know that operation also mobilises liver glycogen and causes a rise in blood sugar. An elevation of the blood sugar persisting for a couple of days should therefore be interpreted as a serviceable reaction which is associated with the adaptation of the organism to trauma. The rise in blood sugar corresponds to the period during which activation of the adrenals and the sympathetic nervous system is seen after surgical trauma (2). It may be produced by the glucocorticoid excreted by the adrenals.

In rabbits affected with alloxan-diabetes the blood sugar elevating effect of Largactil, Atosil and urethane was considerably more transient compared to the reaction in healthy animals. In addition, the rise was followed by a fall even below the original values, for several days' duration. In connection with operation the rise of blood sugar was stronger than merely under the administration of these drugs, but the activation could not, in regard to potency and duration, be compared with the rise observed in healthy rabbits. This more restricted reaction and the subsequent fall of blood sugar, usually below its original level, might be explained so that the test animals had not been balanced with insulin, the stored glycogen therefore being possibly deficient, nor

did the glycogen mobilisation produced by the strain of operation suffice for a more considerable rise of blood sugar. This theory is also supported by the circumstance that the rise of blood sugar was slighter in severe diabetes shan in the milder forms. Since the animals had not been balanced in regard to diabetes, and since alloxan-diabetes may have produced liver damage which is not associated with usual diabetes, these observations as such cannot of course be adopted for the clinic. It must be understood, however, that the observations argue for the exercise of care in the use of insulin during modern anesthesia: preoperative Largactil-Atosil, anesthesia proper without ether. After a comparatively transient elevation, the blood sugar has again a falling tendency. If, by increasing insulin, attempts are made to inhibit the reaction of the organism serving to elevate blood sugar, the effect of insulin will often still continue even while the blood sugar begins to fall, and then hypoglycemic shock may easily be brought about, which may be apt to cause a great deal of difficulties in the patient weakened by surgery.

SUMMARY

The authors have made experimental studies on the effect of chlorpromazin, Atosil, urethane anesthesia and laparotomy on the blood sugar of healthy and alloxandiabetic rabbits (a total of 54 test animals). All these factors had an immediate blood sugar elevating effect on both healthy and diabetic rabbits. The effect of chlorpromazin, Atosil and urethane was fairly transient, the effect of surgery had a longer duration. Healthy and alloxandiabetic rabbits reacted in an entirely different way in so far as in the healthy ones the blood sugar returned to normal after the termination of the experiment, whereas in alloxandiabetic rabbits a fall of blood sugar values below the pre-experimental level occurred immediately after the peak had been reached. This fall persisted for 1—3 days. The alloxandiabetic rabbits used in these tests were not balanced in regard to diabetes.

REFERENCES

1. PASCHKIS, KARL E., RAKOFF, ABRAHAM, and CANTAROW, ABRAHAM: Clinical Endocrinology.
2. PATTERSON, J. W.: J. Biol. Chem. 1950:*183*:81.
3. PATTERSON, J. W., and LAZOROW, A.: J. Biol. Chem. 1950:*186*:141.

DEOXYRIBONUCLEIC ACID IN SPUTUM

STUDIES ON PATIENTS WITH BRONCHIECTASIS, PULMONARY TUBER-CULOSIS AND PULMONARY CARCINOMA

by

ELJAS BRANDER, REINO MÄKITALO and MARTTI TURUNEN

Examination of the sputum plays an essential part in the diagnostics of pulmonary diseases and in evaluating the course of the disease. Particularly scarce attention is however devoted to the chemical analysis of sputum. A search of the literature revealed only few reference to chemical studies of proteins (3, 4). The object of this paper was to study cell nuclei material of sputum by means of determinations of deoxyribonucleic acid (DNA) in certain pulmonary processes.

MATERIAL

This series consisted of 36 patients, with the following diagnostic distribution: bronchiectasis 12, carcinoma of the lung 11, cases of tuberculosis 13, all of the latter far advanced and cavernous. In addition, control analyses were made of 8 normal bronchial secretions from patients on whom aspiration of tracheal mucus was carried out in connection with different abdominal operations. Clinically they were free from sputum, and radiological examination failed to reveal any pathological changes. The amount of sputum per 24 hours was used.

METHODS

Preparative procedure for DNA in sputum based on extraction with strong salt solution according to Mirsky and Pollister (1): The sputum sample was washed in a 0.1 M NaCl solution, to which 0.05 M sodium citrate had been added. The precipitate was then quickly separated by centrifuging and diluted with an approximately equal amount of 1 M NaCl. The thick viscous solution obtained was homogenised by magnetic stirring for 2 hours. After this the solution was deproteinised by using chloroform, to which octyl alcohol in proportion 1 : 3 had been added. In 2 hours of mechanical mixing an emulsion was obtained which was centrifuged (400—4.500 r.p.m.). Three fractions were obtained, of which the upper layer contained the slightly opalescent and viscous sodium deoxyribonucleate. If the upper layer failed to become transparent, ultracentrifuging was used up to 20.000 r.p.m. After centrifugation the upper fraction was analysed in a suitable dilution spectrophoto-
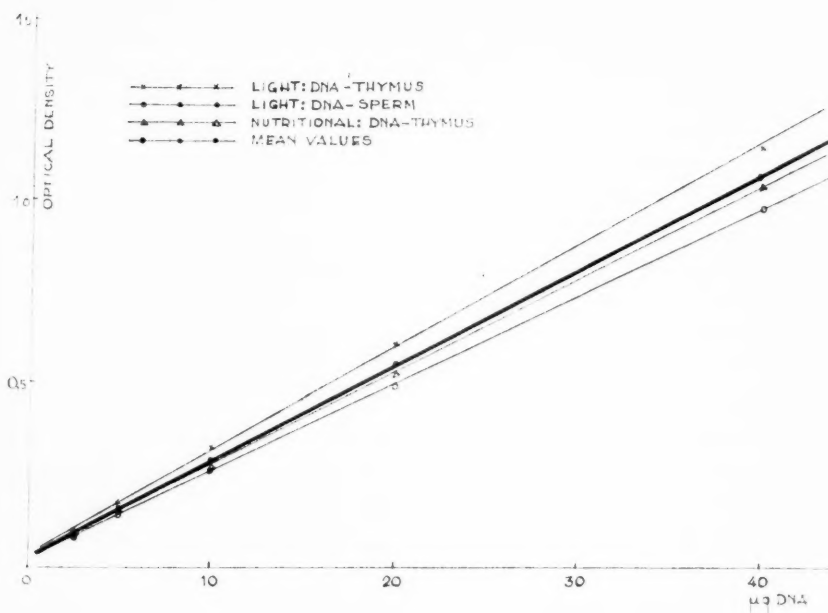
Fig. 1. — Calibration curve for determination of deoxyribonucleic acid concentration.
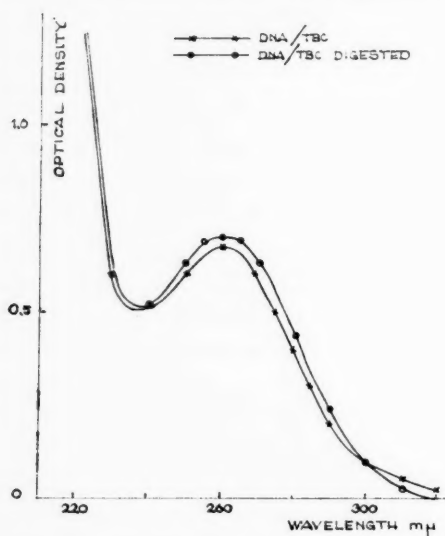
Fig. 2. — Change in ultraviolet light absorption spectrum of sputum deoxyribo-
nucleic acid (tbc) on digestion with crystalline DNase.

metrically on an ultra-violet wavelength of 200—320 mμ (Zeiss
P M Q II), with particular attention to the maximum and minimum
wavelengths. For the blank solution 1 M NaCl was used which was
treated in the same manner as the samples proper with a mixture
of chloroform and octylalcohol. After determination of the
maximum absorption the relative concentration was analysed at
260 mμ from a calibration curve (Fig. 1), which was plotted as
the mean value of three different values analysed with the DNA
preparation (Light: DNA-thymus, Light: DNA-sperm, Nutritional:
DNA-thymus).

Some tests for crude sodium deoxyribonucleate fraction pre-
pared from various sputa:

Prior to the spectrophotometric determination, all sputum
samples were invariably subjected to the Dische diphenylamine
reaction qualitatively. It proved always to be strongly positive.
The typical DNA absorption curve was practically always easily
obtained from the UV absorption curve (Fig. 2). If the maximum
absorption was brought out at 260 mμ in spite of repeated treat-
ment with chloroform-octylalcohol, the sample was discarded. The
absorption minimum, on the other hand, showed considerable varia-
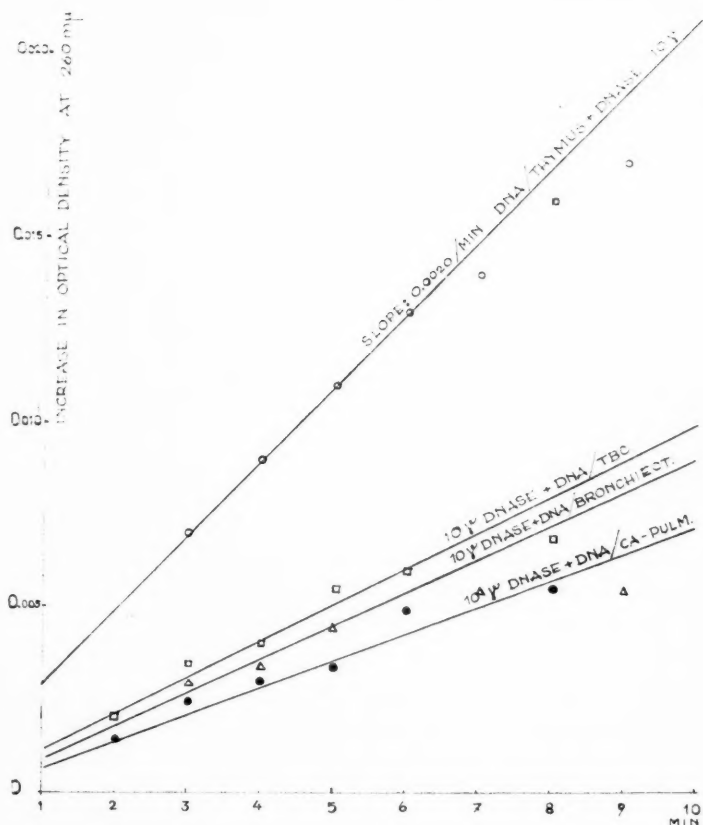
Fig. 3. — Rate of increase of ultraviolet light absorption by deoxyribonucleic acids prepared from various sputa on digestion with crystalline DNase.

tions. Some samples from each diagnostic group were digested by crystalline deoxyribonuclease (Worthington). In spite of the strong NaCl milieu, a marked rise of extinction was invariably obtained (Fig. 3), which, however, was considerably lower than the change of extinction obtained under optimal conditions in a commercial preparation. Under corresponding digestion of samples by crystalline ribonuclease (Worthington) no changes in the extinction could be observed.

The DNA-analyses were performed at random, without knowledge of the diagnosis, and they were invariably carried out by one and the same person.

RESULTS

Table 1 shows the sputum volume per 24 hours, the total mg-amounts of DNA in sputa per 24 hours and mg-amounts per ml of sputum. The means were calculated and by variance analysis compared to mg per ml values in the groups of bronchiectasis, tuberculosis and carcinoma pulmonum.[1]

It was found that the differences between the pulmonary carcinoma group as compared to the tuberculosis and bronchiectasis groups were statistically almost significant (with 97.5% level). On the other hand the differences between these two latter were not significant. In addition dispersion in the carcinoma group was smaller in a significant manner.

DISCUSSION

In the examination of sputa one is faced with the difficulty of isolating and determining a uniform characteristic which could be readily analysed. This study has adopted as such a characteristic the DNA amount in sputa per 24 hours, as a sodium salt in concentrated salt solution. Bronchial secretion would naturally supply a more accurate picture of what is expelled from the lungs. Yet by aspiration at bronchoscopy will only yield the mucus which is present there at the time.

DNA itself was not finally isolated by precipitation, nor were its chemical and physical characteristics been subjected to a closer examination. Endeavours were made above all to aschieve biochemical collective analysis, and for this purpose the UV spectrum of the specimen was analysed after a suitable and simple purification process, in order to find out the absorption maximum. It was attempted in addition to clarify the nature of the substance qualitatively. The Dische diphenylamine reaction was carried out and efforts were made, in association with digestion by crystalline enzymes, to perform a spectrophotometric study of the extinction changes, which is proved to be specific for nucleic acids (2). Taking into consideration the isolation and purification procedures, as well as the identification tests performed, the results indicate that

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[1] The authors express their gratitude to Prof. Olli Lokki Ph. D. for the statistico-mathematical treatment of the material.

TABLE 1

Bronchiectasis			Pulmonary Tuberculosis			Pulmonary Carcinoma			Controls		
Sputum Volume ml	Total DNA mg	DNA mg/ml	Sputum Volume ml	Total DNA mg	DNA mg/ml	Sputum Volume ml	Total DNA mg	DNA mg/ml	Bronchial Secretion Volume ml	Total DNA mg	DNA mg/ml
160	62.4	0.39	40	56.9	1.42	20.0	5.2	0.26	2	0.12	0.06
60	124.8	2.08	40	86.0	2.15	10.0	3.3	0.33	2	0.02	0.01
100	248.0	2.48	23	21.3	0.93	35.0	19.9	0.57	2	0	0
57	234.8	4.12	25	10.8	0.36	65.0	10.4	0.16	2	0	0
35	32.2	0.92	40	46.4	1.16	30.0	15.0	0.50	2	0	0
180	180.0	1.00	20	10.1	0.50	7.5	2.6	0.35	2	0.11	0.06
30	158.4	5.28	15	12.1	0.94	40.0	0	0	2	0.09	0.05
30	120.0	4.00	25	8.0	0.31	5.0	0	0		0	0
50	102.0	2.04	30	60.0	2.00	45.0	0	0			
180	108.0	0.90	15	7.2	0.48	10.0	7.8	0.78			
35	21.0	0.60	35	35.3	1.01	10.0	5.6	0.56			
160	80.0	0.50	100	168.0	1.68						
			25	177.5	7.10						
	Mean 122.6	Mean 2.03		Mean 53.8	Mean 1.54		Mean 6.9	Mean 0.32		Mean 0.04	Mean 0.029
		Standard deviation of mean 0.48			Standard deviation of mean 0.50			Standard deviation of mean 0.08			

success has been achieved in bringing out the DNA fraction with sufficient accuracy.

By the method employed we have been able to isolate considerable amounts of DNA in two mainly inflammatory diseases, pulmonary tuberculosis and bronchiectasis. On the other hand, the corresponding values obtained from the sputum of patients with carcinoma of the lung were markedly lower. It must be noted, however, that all the carcinoma cases of our series were more or less incipient and without softening. If there is an abscess or cavity formation in the carcinoma focus, it is to be expected that the DNA content in the sputum will rise. To the contrary for advanced cases were selected from tuberculosis patients.

The DNA determination method here employed is relatively simple, and the results obtained with it yielded fairly marked quantitative differences. It would therefore appear suitable for a further investigation of sputum.

SUMMARY

A spectrophotometric analysis of 44 sputum samples was performed by a method based on the isolation of deoxyribonucleic acid from the sputum in a concentrated NaCl extract. The total amounts of DNA in mg per 24 hours and in mg per ml of sputum were calculated in bronchiectasis, pulmonary tuberculosis and carcinoma of the lung. A marked difference was demonstrated in this material between the DNA content of sputum of bronchiectasis and tuberculosis patients on the one hand, and of carcinoma patients on the other.

REFERENCES

1. CHARGAFF, E., and DAVIDSON, J. N.: The Nucleic Acids I & II. Academic Press. New York. 1955.
2. KUNITZ, M.: J. Gen. Physiol. 1950:33:357.
3. NYLANDER, P. E. A., HALONEN, P. I., KÄRKKÄINEN, V. J., and TURUNEN, M.: Ann. Chir. et Gyn. Fenniae. 1954:43:suppl. 5.
4. WARFVINGE, L. E.: Acta Med. Scand. 1955:153/1:49.

STUDIES IN THE VISCOSITY OF BRONCHIAL MUCUS

by

P. LEIKKOLA, K. HARTIALA, S. VIIKARI and U. SIIRALA

The digestion of mucous substances of various origins has been the subject of some studies (2, 6, 7, 8, 9, 11, 12, 13, 14, 16). The proteolytic nature of the depolymerization of mucus secreted by the duodenal (Brunner) and gastric glands has been well established (2, 8, 11, 12, 13). This paper is a report of some studies carried out on bronchial mucus in human subjects. It was reasoned that a comparative study of the behaviour of various mucous specimens towards factors known to affect the physical and chemical nature of other mucous substances might add to our knowledge concerning the functions of the mucous barrier system in different regions of the organism.

MATERIAL AND METHODS

The mucus was collected directly from the bronchi in connection with operative procedures. The patients suffered from pulmonary tuberculosis or bronchiectasis. Samples of bronchial carcinoma were not included in the series. Specimens containing blood were discarded since blood itself is known to contain proteolytic enzymes (1, 10, 15, 18). In a few cases the mucus was aspirated into collecting flasks during bronchoscopy.

From previous studies it is known that the loss of viscosity can be prevented by cooling the samples. For this purpose the mucus was immediately placed into a glass container immersed in ice. It was kept in this condition until the actual viscosity studies were performed.

The Höppler viscosimeter was used in this work. A special tube rendered this apparatus suitable also for microdeterminations. The apparatus was connected to a constant temperature water bath. All studies were performed at 37°C.
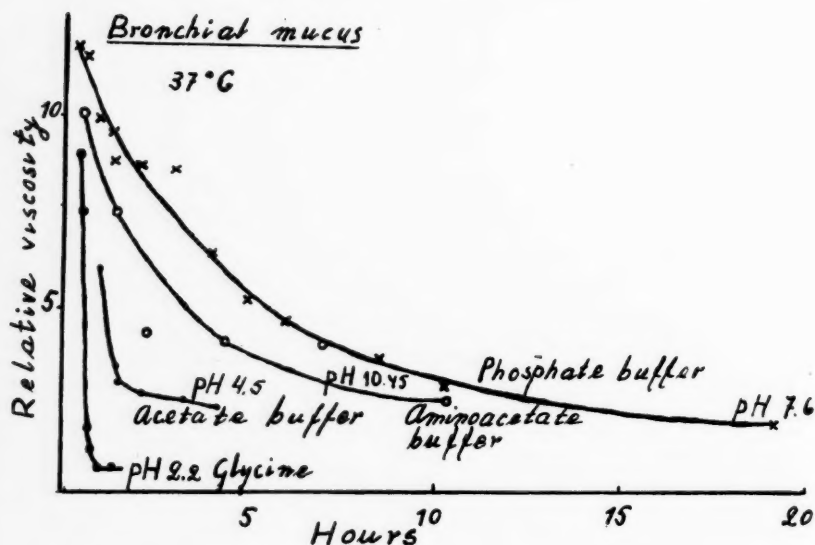
Fig. 1. — Loss of viscosity of bronchial mucus at various pH levels.

In order to stabilize the conditions, the mucus samples were diluted with buffer solutions. In most of the studies the Sörensen phosphate buffer was used. For lower pH values acetate and glycine-HCl buffers were used. It was thus possible to study the changes in viscosity within the wide range of pH from 2.2 to 10.5.

One volume of mucus and two volume of the buffer solution were used; the total amount of reaction mixture was 2 or 3 ml. The results are expressed im terms of relative viscosity with distilled water as unity.

The effect of enzymes on the viscosity was studied by adding the test substances after a few control readings were obtained.

RESULTS

The effect of the pH of the reaction mixture on the spontaneous change of native bronchial mucus is illustrated in Fig. 1. It appears that at 37°C the loss of viscosity takes place within a few hours. The exceptionally rapid changes in the lower pH ranges are of special interest.

The effect of the proteolytic enzymes trypsin and chymotrypsin is illustrated in Fig. 2. Whereas the spontaneous loss of viscosity takes several hours, at pH 7.6 these enzymes produced a change in a much shorter time. The rapid action of cysteine is also apparent.
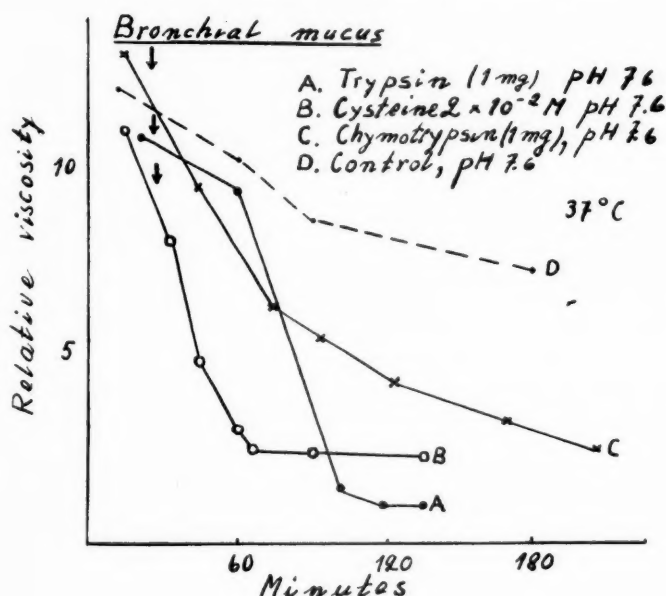
Fig. 2. — Effect of various enzymes and cysteine on the loss of viscosity in bronchial mucus.

It should be noted that under these experimental conditions the mucus changed into a waterlike substance.

DISCUSSION

The most important result of the experiments was that they provided further evidence concerning the proteolytic nature of the depolymerization process associated with the decrease in viscosity of the mucus. In this respect bronchial mucus does not differ from duodenal or gastric mucus. The only difference lies in the fact that at lower pH levels bronchial mucus loses its mucous nature extremely rapidly. The experiments do not provide support for the prevailing theories concerning the cause of this difference.

These low pH ranges suggest activity on the part of some katepsin enzymes which act in an acid environment (4, 5, 17). These enzymes are known to be widely distributed in animal tissues, also in the lungs (3).

It has also been shown that some tissue katepsin enzymes are activated by reducing agents such as cysteine. We have also found that cysteine accelerates the disintegration of mucus.

SUMMARY

The loss of viscosity of native human bronchial mucus has been studied under various pH conditions.

It was found that the spontaneous loss of viscosity depended greatly on the pH of the reaction mixture. The most rapid change took place under acid conditions.

In accordance with previous findings concerning mucous substances, the decrease in the viscosity of bronchial mucus was found to be greatly enhanced by proteolytic enzymes.

Cysteine also greatly accelerated the rate of decrease in viscosity.

REFERENCES

1. ABDERHALDEN, E., and HANSON, H.: Fermentforschung 1937:15:382.
2. ANDERSON, R. K., and FARMER, C. J.: Proc. Soc. Exp. Biol. N.Y. 1934—35:32:21.
3. FRUTON, J. S.: J. Biol. Chem. 1946:166:721.
4. FRUTON, J. S., and BERGMANN, M.: J. Biol. Chem. 1939:130:19.
5. FRUTON, J. S., IRVING, G. W. JR., and BERGMANN, M.: J. Biol. Chem. 1941:138:249.
6. GLASS, G. B. J.: Rev. Gastroenterol. 1949:16:687.
7. GLASS, G. B. J., and BOYD, L. J.: Gastroenterology 1949:12:821.
8. GLASS, G. B. J., and BOYD, L. J.: Gastroenterology 1949:12:1.
9. GLASS, G. B. J., PUGH, B. L., GRACE, W. J., and WOLF, S.: J. Clin. Invest. 1950:29:12.
10. GROSSMANN, W., and HEYDE, W.: J. physiol. Chem. 1950:188:69.
11. HARTIALA, K., and GROSSMAN, M. I.: J. Biol. Chem. 1952:195:251.
12. HARTIALA, K., IVY, A. C., and GROSSMAN, M. I.: Am. J. Physiol. 1950:162:110.
13. JANOWITZ, H. D., and HOLLANDER, F.: Gastroenterology 1954:26:582.
14. MAHLO, A., and MULLI, N.: D. Med. Wochenschr. 1934:60:1632.
15. MASCHMANN, E.: Biochem. Zschr. 1941:308:359.
16. PEKELHARING, C. A.: J. physiol. Chem. 1902:35:8.
17. SUMNER, J. B., and MYRBÄCK, K.: Acad. Press. Inc. Publishers. New York 1951.
18. WEIL, L., and RUSSEL, M. A.: J. Biol. Chem. 1938:126:245.

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CORRIGENDUM

In der Arbeit von R. Takkunen und J. Alberty: Histamin-antagonistische Wirksamkeit ...., Vol. *34*, 1956, müssen auf Seite 217 in Tabelle 2 die Worte

Antazolin m.sulf.   und Pyrilamin mal., sowie
Atropin sulf.   und Diphenhydr. hydrochl.

gegeneinander ausgetauscht werden.   .

REQUEST

The Hungarian Laboratory for Blood Clotting and Practical Examination of Muscles (Head: E. Szirmai, M.D.) asks for reprints concerning practical myology.

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COENZYME A IN LIVERS OF RATS TREATED WITH GROWTH HORMONE (STH)

by

J. JÄNNES, R. KÄRÄVÄ and T. LUUKKAINEN

The influence of the growth hormone (STH) on carbohydrate and fat metabolism has been a subject of wide interest during the last few years. As Greenbaum (3, 4) postulated, the hypophyseal growth hormone (STH) has an inhibitory effect on the synthesis of fats in liver slices. According to Lynen and Ochoa (7), the synthesis or breakdown of fatty acids in livers depends on the ratio DPN/DPNH. Changes in the amount of reduced coenzyme I DPNH gives the direction to the ›fatty acid cycle»: it determines whether synthesis or degradation of long carbon chains will occur.

Greenbaum (3) observed in his experiments that STH increased the amount of reduced coenzyme I (DPNH) and in this way accelerated the breakdown of fatty acids.

Engel (2) pointed out that the action of STH on the terminal oxidation of the two carbon fragments was characterised by acceleration of the breakdown of acetoacetyl-coenzyme A. According to him, the explanation for the increase of the ketone bodies in the blood after STH treatment is the stimulation of the deacylase enzyme, which splits acetoacetyl-coenzyme A into acetoacetate and coenzyme A. On the other hand, many inhibitory effects on the terminal metabolism of acetate via the tricarboxylic acid cycle have been demonstrated. The well known stimulation of protein synthesis produced by STH is explaned by the accelerated conversion of pyruvate and oxaloacetate to amino acids.

The growth hormone (STH) has, according the recent point of view, a great influence on many intermediary reactions in which coenzyme A is involved.

The effect of several hormones on the coenzyme A (Co A) of organs has been investigated using rats as test animals. Tabachnick and Bonnycastle (9) observed that L-thyroxine caused in thyreodectomised animals a definite increase in the Co A content in different organs of the rats. Ringler and Leonard (8) were able to note that L-thyroxine, cortisone, adrenocorticotropic hormone (ACTH) and hypophyseal growth hormone (STH) were capable of increasing the Co A content of livers in hypophysectomised rats. Using intact animals they noted that a single dose of STH was able to decrease the Co A content of the livers by about 20 per cent.

Because of the short duration of the experiments of Ringler and Leonard, the writers describe in the following some series of tests in which the treatment with STH was of a longer duration, the test animals being allowed to become adapted to a longer administration of STH.

According to newer investigations of Luukkainen (6), STH may have a more prominent effect on the metabolism by pregnant rats than by non-pregnant controls. Therefore a series of tests were added to clarify the effect STH on the Co A content in the livers by pregnant rats.

<div align="center">MATERIAL AND METHODS</div>

In all the tests female intact rats of Wistar strain were used. The weight of the animals varied between 130—150 gm. The diet used contained maize, wheat bran, casein, NaCl and $CaCO^3$. The animals were fed *ad libitum*. The test animals were divided into four groups as follows:

1) Normal control animals
2) Normal animals treated with STH
3) Pregnant control animals
4) Pregnant animals treated with STH.

The STH preparation used was manufactured by Nordisk Insulinlaboratorium, Malmö, Sweden, under the name of »Somacton». The rats were treated with a 11 ng acting »somacton» pre-

paration for 28 days and the daily dosage was 30 tibia units administred intramuscurally. The preparation »somacton» had been found to be effective in other tests. After 12 hours' fasting the animals were killed by decapitation, the pregnant animals immediately after delivery. The livers were quickly removed and 1 g of the liver tissue was suspended in 4 ml of water and boiled on a water bath. From this »Kochsaft» the assay of Co A was carried out. The transacetylase assay of Kaplan and Lipmann (5) was used in these experiments. The pigeon liver enzyme, prepared by the authors, was able to acetylate 70 per cent of sulfanilamide in the reaction mixture. The adenosinetriphosphoric acid (ATP) used was manufactured by Light & Co.

The authors had in this work no possibility to operate with pure Co A standards. A concentrated »Kochsaft» of rat liver was used as standard and the half maximum level of acetylation was considered as 1 unit of Co A. Because of the lack of a pure standard. The values presented here have a relative reliability. For this reason a normal control series was carried out in every assay series at the side of STH treated material.

The transacetylase assay has according to Boxer and co-workers (1) the disadvantage, that the addition of ATP may synthesise new Co A from split products. The values obtained by this method are therefore higher than the results reported by other methods for Co A assay. The values obtained in these tests varied between 45 and 76 units of CoA in 1 gm of rat liver.

RESULTS

	Normal Control Animals	Normal Animals Treated with STH	Pregnant Control Animals	Pregnant Animals Treated with STH
Number of cases ..	6	7	6	3
Co A units in 1 g of liver (average) ..	57	65	65	60

The acetylation readings with the »Kochsaft» of livers showed no significant differences in any group.

In the above mentioned experiments the STH preparation was given intramuscurally and the it had a long acting effect. Because

of the possible adaptation of the test animals to long acting
»somacton», another series with a short acting preparation were
also carried out.

In these tests to three female non-pregnant rats were given
during 3 days 80 tibia units (0.36 mg) daily intraperitonally. The
whole amount of injected »somacton» was about 1 mg per rat.

After 24 hours' fasting the animals were decapitated and the
livers were investigated in the usual manner. Two normal female
rats served as controls.

	Control Rats (2)	STH-Treated Rats (3)
Co A units in 1 g of liver (average)	70	67

The differences obtained between the STH treated animals
and the controls were very little. Therefore the authors could not
confirm the results of Ringler and Leonard (8). After our results
the STH treatment has no effect on the Co A content in the livers
of intact rats.

SUMMARY

The writers studied the effect of somatotropic growth hormone,
»somacton», on the coenzyme A levels in the livers of pregnant and
non-pregnant rats. No noteworthy differences could be detected
between the STH-treated animals and the controls.

LITERATURE

1) BOXER, G. E., SHONK, C. E., GILFILLAN, E. W., EMERSON, G. A., and
    OGINSKY, E.: Archives Biochem. Biophys. 1955:59:24.
2) ENGEL, F.: Hypophyseal Growth Hormone. — Mac Graw-Hill, Phila-
    delphia 1955 page 344.
3) GREENBAUM, A. L.: Biochem. J. 1953:54:400.
4) GREENBAUM, A. L.: Hypophyseal Growth Hormone. — Mac GRAW-HILL,
    Philadelphia 1955 page 340.
5) KAPLAN, N. O., and LIPMANN, F.: J. Biol. Chem. 1948:174:37.
6) LUUKKAINEN, T.: Peronal Report to be published.
7) LYNEN, F., and OCHOA, S.: Biochem. Biophys. Acta 1953:12:299.
8) RINGLER, I., and LEONARD, S.: Endocrinology 1954:55:363.
9) TABACHNICK, I., and BONNYCASTLE, D.: Nature 1953:172:400.

EFFECT OF DINITROPHENOLS, SOME NARCOTICS, AND THIOUREA ON THE RESPIRATORY METABOLISM OF WHITE MICE

by

KARI LAGERSPETZ and LEA VIILI

The metabolic effects of the dinitrophenols were first demonstrated in 1932 by Magne, Mayer & Plantefol (13) and simultaneously by Cutting & Tainter (3). Since then, a copious literature has accumulated, dealing with the respiratory responses of various organisms and tissues to the dinitrophenols and their related compounds (5, 18) and especially with the respiratory effects of the most powerful substance of this group, 2,4-dinitrophenol (17). The active form and the biochemical mechanism of action of 2,4-dinitrophenol have been the subject of many studies (4, 14, 10, 12, 8, 7). Thus it has been suggested that the dinitro compounds reversibly uncouple the phosphorylation and oxidation processes, whose participation is essential for the energy-requiring activities within the organism.

In this study, an attempt is made to present the effects of dinitrophenols, some narcotics, and thiourea on the respiratory metabolism of mice. As the results obtained will serve as a comparation basis for a study about the combined effects of 2,4-dinitrophenol and narcotics or thiourea, the experimental results presented here will be discussed in that context (11).

MATERIAL AND METHODS

1. Test Animals. — The white mice were fed on a mixed diet consisting of bread, oats, milk, and some vegetables. Most of the animals used in the experiments were males, but occasionally females were also used. Since control readings were taken at the beginning of each experiment as described later, any changes in the respiratory metabolism of the females due to the oestrous cycle could be eliminated.

2. Measurement of O_2 Consumption, CO_2 Output, and R.Q. — The apparatus used in the experiments was a slightly modified Haldane-Kendeigh apparatus (6, 9, 15). The air current was drawn in by means of a suction pump through a system consisting of 1) a Drechsel gas washing bottle containing 60% KOH, which absorbed the carbon dioxide from the air current, 2) an U-tube containing silica gel with moisture indicator (May & Baker) for drying the air, 3) an animal chamber of glass, closed with a rubber stopper through which a thermometer was inserted, 4) a series of five U-tubes, the first containing silica gel for absorbing the water produced by the test animal, the second containing soda lime (May & Baker or Merck) which absorbed the carbon dioxide produced by the animal, the third containing silica gel for the absorption of water escaping from the second tube with the air current, the fourth, containing soda lime, and the fifth, containing silica gel, both acting as controls for the prevailing absorption capacity of the former tubes and at the same time collecting the moisture which might be brought into the apparatus by a sudden change of pressure (this did not happen in our experiments). After passing through the U-tubes, the air current flows through a T-stopcock, and to the suction pump. All connections between the different tubes and other parts of the apparatus were of thick-walled rubber tubing.

The amount of CO_2 produced by the animal in a given time was obtained by weighing together the second and third U-tubes in the series before and after the experiment. The increase in the weight of these tubes gave the CO_2 produced by the animal. The amount of O_2 consumed was calculated by subtracting the weight loss of the animal during the experiment from the sum of the CO_2 output and H_2O production, the latter being obtained from the increase in weight of the first U-tube in the series. A detailed account of

the calculations is given by Muralt (15). The R.Q. was calculated in the usual way and the O_2 consumption expressed in ml per gram of body weight per hour (ml/g/h). The accuracy of the weighings was at least 0.5 mg.

In each experiment, the respiration of the test animal was first measured during two successive 15-minute intervals. The O_2 consumption and R.Q. values obtained from these measurements served as controls and are presented in columns 1 and 2 in the following tables. Then the substances to be tested were injected into the animal, and two successive respiration measurements immediately performed. The animal was then removed from the animal chamber and transferred to a container for 15 minutes, and, after this, the respiration during a 15-minute period again measured. This procedure was repeated. Accordingly, the values in columns 3, 4, 5, and 6 represent the O_2 consumption and R.Q. values for periods 0—15, 15—30, 45—60; and 75—90 minutes after the injection. A period of 15 minutes was found to be the most satisfactory, because of the rapid effect of the substances tested. Two series of U-tubes, used interchangeably, allowed the measurements to be continuous.

3. *Sources of Error in the Respiration Measurements.* — When using a Haldane-Kendeigh apparatus in respiration measurements, great care must be taken that the absorption capacity of the absorbents used in the apparatus (KOH, silica gel, soda lime) does not diminish. The absorption capacity of the KOH was checked by observing the amount of precipitate in the gas washing bottle. The silica gel contained a moisture indicator, the colour changes of which were watched. By weighing the fourth and fifth U-tubes in the series, the absorption capacity of the soda lime could be tested. To be sure that there was no leakage in the apparatus, control experiments of 15 minutes' duration without animals were performed before the majority of ordinary experiments.

Whenever the test animal urinated or passed faeces during the experiment, this caused the R.Q. to rise above 1. Such experiments were interrupted and the readings discarded.

The temperature of the animal chamber was watched by means of the inserted thermometer, partly to render it possible to avoid sudden changes in temperature resulting in condensation of water inside the apparatus. These could be prevented if the velocity of

the air current was kept sufficiently high and constant. The tem-
perature was also followed so as to obtain the mean temperature
during the entire experiment. The environmental temperature
affects the metabolism of even homoiothermic animals, and therefore
the metabolic levels determined in experiments performed at
different temperatures are not directly comparable. Since the first
two 15-minute periods during each experiment served as controls,
and the temperature differences during an experiment were negli-
gible, the respiration intensity after the injections could be compared
with the two slightly earlier control values. The temperature of the
animal chamber varied in the different experiments between 18°C
and 26°C.

Both the weighing of the test animal at intervals of 15 minutes
and the injections per se might have a disturbing effect. There-
fore, and in order to obtain the normal variation range, control
experiments were performed in which 0.2—0.3 ml of physiological
saline was injected subcutaneously. The results of these control
experiments are presented in Table 1. The injection did not alter

TABLE 1

THE EFFECT OF AN INJECTION OF PHYSIOLOGICAL SALINE ON THE RESPIRATORY
METABOLISM. COLUMNS 1 AND 2 GIVE THE CONTROL VALUES OBTAINED DURING
TWO 15-MINUTE PERIODS BEFORE INJECTION. THE VALUES IN COLUMNS 3, 4, 5,
AND 6 REPRESENT THE RESULTS FOR MEASUREMENT PERIODS 0—15, 15—30,
45—60, AND 75—90 MINUTES AFTER INJECTION. THE FIRST FIGURE IN EACH
COLUMN INDICATES THE O_2 CONSUMPTION (ml/g/h) AND THE SECOND, THE R.Q.

Body Weight g	1		2	inj.	3		4		5		6	
21.5	5.40	0.69	5.11	0.69	3.36	1.08	4.32	0.69	4.40	0.63	3.73	0.66
24.2	4.52	0.73	2.95	0.63	4.86	0.81	5.51	0.72	4.18	0.71	6.40	0.72
24.5	5.46	0.74	5.65	0.69	5.50	0.78	3.43	0.86	4.07	0.77	3.65	0.70
24.9	3.50	0.73	4.18	0.98	4.05	0.61	6.64	0.63	3.00	0.63	4.44	0.83
25.8	4.70	0.83	5.87	0.82	4.35	0.94	4.48	0.65	4.20	0.74	3.77	0.62
26.5	4.52	0.76	4.20	0.63	3.60	0.76	3.21	0.78	3.91	0.73	3.46	0.60
26.9	4.50	0.80	3.96	0.65	3.79	0.81	5.08	0.63	4.97	0.70	4.98	0.67
28.3	4.77	0.68	4.01	0.62	4.43	0.73	3.60	0.62	3.72	0.69	3.20	0.69
25.3	4.64	0.74	4.49	0.71	4.24	0.81	4.53	0.70	4.05	0.70	4.20	0.68

the O_2 consumption or R.Q. of the test animals. The O_2 consump-
tion is slightly higher during the first periods. This obviously
depends upon the greater activity of the mice in the beginning of the
experiment. The results of ordinary experiments were compared

with the values obtained in experiments in which the animals, too, were weighed and therefore possibly disturbed every 15 minutes.

The metabolic activity of homoiothermic animals, at least, is proportional to the area of body surface. The O_2 consumption as expressed in ml per hour per gram of body weight is, accordingly, greater in small-sized individuals with a relatively greater surface area than in larger ones. Thus expressed only the metabolism of animals of roughly the same size can be compared. Therefore only animals weighing 20—28 g were used in the experiments. Their O_2 consumption values were approximately equal (see Table 1).

Even the alimentary state of the animals seemed to affect the results. Wallgren (20) studied this source of error in his experiments with Emberiza spp. Wallgren concluded that after a fast of 4 hours' duration the after-effects of a meal on respiration were abolished. Four mice were allowed to fast for 30 hours and the O_2 consumption and R.Q. of each mouse measured immediately after the meal and 1, 2, 3, 5, 10, 15, 20, and 30 hours after the meal always during four 15-minute periods. The fasting began in all cases at 8 o'clock in the morning. The results are summarized in Table 2. The R.Q.

TABLE 2

THE EFFECT OF FASTING ON THE RESPIRATORY METABOLISM. THE VALUES FOR OXYGEN CONSUMPTION AND R.Q. ARE MEANS OF FOUR SUCCESSIVE MEASUREMENTS (15-MIN. PERIODS) CARRIED OUT WITH EACH OF FOUR TEST ANIMALS

Time Fasted in Hours	Mean Oxygen Consumption ml/g/h	Mean R.Q.
0	5.01	0.99
1	3.89	0.88
2	4.64	0.74
3	5.09	0.78
5	4.65	0.68
10	4.66	0.67
15	4.28	0.71
20	4.51	0.78
30	4.41	0.75

was high during the two first hours after the meal. There were no clear-cut postprandial or diurnal changes in the O_2 consumption. In the experiments, the mice were accordingly allowed to fast for at least three hours before the respiration measurements were begun.

One point worth mentioning is that in the respiration measurements carried out with the Haldane-Kendeigh apparatus, the test animals are in air with a low relative humidity. This renders these experiments not directly comparable with results obtained with other methods. In addition, it must be pointed out that the water which is absorbed by the silica gel includes water eliminated through body surfaces other than the lungs (transpired water).

4. *Solutions and Injections.* — The dinitrophenol (DNP) solutions were prepared by a modification of the method of Clowes & Krahl (2). The DNP was dissolved in 0.3—0.5 ml of 1 M NaOH in a measuring flask, distilled water added, and, after the DNP was completely dissolved, more water added; the pH, which was 8.5—9, was adjusted to about 7.5 with HCl, and the flask filled to the graduation mark with distilled water. The DNP was thus present in the solution as sodium dinitrophenolate. The amounts injected into the mice were expressed as the amount of originally free DNP in solution per 100 g of body weight (mg%). The DNP:s used were manufactured by BDH. Other solutions used were nembutal (pentobarbital) (Abbott) 0.4%, ethyl urethane (Merck) 10%, and thiourea (Merck) 0.1% aqueous solutions. Solutions were injected subcutaneously into the back of the test animal.

The same animal was only used in experiments at most twice a week. The two values obtained before the injections in each experiment served as controls for any change in metabolic level caused by the earlier injections.

5. *Statistical Methods.* — When comparing the O_2 consumption before and after injections, the means of the O_2 consumption with standard errors during the second control period (column 2 in the tables) and during that experimental period in which the results diverged most from the mean control values, were compared, using the t-test. The period during which the O_2 consumption was most affected by the substance injected was usually that between 15 and 30 minutes after the injection (column 4 in the tables). Some idea of the duration of the effect was gained by calculating the significance of the difference between O_2 values during the periods 15—30 and 75—90 minutes after the injection (columns 4 and 6). In the calculations, the standard methods presented by Bonnier & Tedin (1) were followed.

EXPERIMENTAL RESULTS

1. Effect of Dinitrophenols on Respiratory Metabolism. a. 2,4-Dinitrophenol. — When 2,4-DNP was used in concentrations of 0.08, 0.1 or 0.6 mg%, neither the respiration nor the behaviour of the test animals was affected. 1 mg% of DNP rendered the animals less active and increased the rate of the respiratory movements in the animals, although no effect on the rate of oxygen consumption was observed. A slight increase in the O_2 consumption was found when 1.5 mg% DNP was injected into the test animals, the other effects being the same as for the previous concentration. 2 mg% DNP clearly stimulated the respiratory metabolism, as can be seen from Table 3. The effect of DNP disappeared in about

TABLE 3

THE EFFECT OF 2 MG% 2,4-DNP INJECTION ON THE RESPIRATORY METABOLISM. COLUMN 7: VALUES FOR A MEASUREMENT PERIOD 105—120 MINUTES AFTER INJECTION. FOR OTHER EXPLANATIONS, SEE TABLE 1

1		2	inj 3		4		5		6		7	
5.67	0.74	5.00	0.71	6.53	0.71	10.00	0.64	7.52	0.67	7.45	0.67	4.40	0.92
6.20	0.69	5.03	0.81	5.82	0.72	7.40	0.65	5.50	0.76	4.29	0.66	5.26	0.73
6.44	0.68	5.30	0.63	5.89	0.76	7.67	0.69	6.55	0.75	6.50	0.72	5.66	0.70
4.61	1.12	4.08	1.17	9.31	0.67	6.42	0.76	7.66	0.76	5.25	0.82		
4.90	0.81	5.39	0.77	8.32	0.76	5.87	0.77	7.72	0.75	8.57	0.64		
3.21	0.74	3.88	0.98	6.88	0.78	8.36	0.75	7.13	0.71	6.73	0.78		
5.88	1.33	5.70	0.69	5.53	0.88	7.75	0.65	6.01	0.76	6.34	0.69		
4.95	0.77	3.75	0.65	7.09	0.72	6.56	0.80	7.10	0.73	7.11	0.72		
5.20	0.70	5.06	0.72	6.22	0.79	8.26	0.60	6.89	0.64	6.41	0.66		
5.22	0.84	4.78	0.79	6.84	0.75	7.58	0.70	6.89	0.72	6.51	0.70	5.10	0.78

two hours. The test animals often urinated 1.5—2 hours after the injection and their urine was of a bright yellow colour, which can probably be attributed to DNP or its breakdown products, which are rapidly eliminated from the body (16). The difference between the oxygen consumption means in columns 2 (4.78 ± 0.23 ml/g/h) and 4 (7.58 ± 0.41 ml/g/h) is significant (t = 5.21, P = 0.001). This concentration was chosen for further studies on the combined effects of DNP and other substances, on account of its clearly stimulating effect on respiration, without any apparent harmful sequelae. At higher concentrations, the lethal effects of DNP appeared. The maximal stimulation with 2.5 mg% was somewhat

higher than with 2 mg%, but two out of three animals tested
died during the experiment (30 and 60 minutes respectively after
the injection). The effect of 3 mg% was the same as that of the
previous concentration. All three test animals into which 3.5 mg%
of DNP was injected died during the experiment (one of these ani-
mals survived for 15 minutes, the other two for an hour). The
greatest individual oxygen consumption values were obtained with
concentrations between 2.5 and 3.5 mg%. The concentrations
4 mg% and 8 mg% were highly toxic, and the animals died 20, 15,
and 10 minutes, and with 8 mg%, even 5 minutes after the admi-
nistration of DNP.

At concentrations lower than 3 mg% the DNP did not affect
the respiratory quotient. For 2 mg%, the R.Q. values are presented
in Table 3. Higher concentrations (3.5 mg% or more) increased the
R.Q. This can be seen from Table 4, in which the O_2 consumption

TABLE 4

THE EFFECT OF 3.5 MG % 2,4-DNP INJECTION ON THE RESPIRATORY METABOLISM.
FOR THE SURVIVAL TIME OF THE TEST ANIMALS, SEE THE TEXT. FOR OTHER EX-
PLANATIONS, SEE TABLE 1

1		2	inj.	3		4		5	
6.40	0.64	5.03	0.66	3.68	0.90	7.81	0.82	8.76	1.20
4.63	0.66	4.17	0.63	7.83	0.88				
4.81	0.66	4.33	0.63	7.45	0.87	5.62	0.96	5.65	0.81
5.28	0.65	4.51	0.64	6.32	0.88	6.71	0.89	7.20	1.00

and R.Q.'s for the test animals which had received 3.5 mg% DNP
are presented. With higher concentrations, R.Q. increased from
0.73 to 0.93 in mean.

b. *2,6-Dinitrophenol.* — When 2,6-DNP was used in concentra-
tions of 2, 3, 4, 5, 6, and 8 mg%, no effect on O_2 consumption or
R.Q. was noticed. The activity of the test animals decreased mar-
kedly at all concentrations used. The concentrations 5—8 mg%
were lethal. The test animals were gasping, and at lethal concentrat-
ions the length of the intervals between respiratory movements
was extended, until after violent convulsions the animals became
stiff.

c. *2,5-Dinitrophenol.* — Some tests were also performed with
2,5-DNP. The concentrations used were 1, 2, 3, and 4 mg%. The

activity of the animals was decreased, but there were no noticeable effects on the O_2 consumption, the R.Q., or the rate of respiratory movements. Swelling was noticeable in the orbital region, and some kind of liquid dripped out of the eyes. This bothered the animals, and they therefore often rubbed their eyes with their forelegs. The concentrations used were not lethal.

2. Effect of Some Narcotics and Thiourea on Respiratory Metabolism. — *a. Nembutal* ($NaC_8H_{11}N_2O_3$) is known to decrease the respiratory metabolism (19). The narcotic effect of nembutal began about 15 minutes after the injection, and sometimes after some convulsions. The measurement of the respiratory metabolism was begun 15 minutes after the injection. The concentration used was 50 mg per kilogram of body weight. The difference between the last control value (4.72 ± 0.24) and the lowest O_2 consumption value (30—45 minutes after the injection, 2.26 ± 0.35) is clearly significant (t = 5.79, P = 0.001). The difference between the lowest oxygen consumption value and the oxygen consumption during the period between 90 and 105 minutes after the injection (3.65 ± 0.45) shows an increase in O_2 consumption, although it has not yet reached the normal level. In these experiments, the control R.Q. means were 0.74 (first control period) and 0.72 (second control period). The R.Q. decreased after the injection: the means were 0.60 (15—30 minutes after the injection), 0.60 (30—45), 0.55 (60—75), and 0.57 (90—105 minutes after the injection).

b. Ethyl urethane (ethyl carbamate) was used in eight experiments in concentration of 1 g per kg of body weight. It seemed not to possess any narcotic effect or any effect on the oxygen consumption or on the R.Q.

c. Thiourea was used in eight experiments in concentration of 1 mg%. It had no effect on the activity, oxygen consumption, or R.Q. of the test animals.

SUMMARY

1. The respiratory metabolism of adult white mice has been studied using the method of Haldane and Kendeigh. The sources of error in this method are discussed.

2. The effect of different concentrations of 2,4-dinitrophenol on the oxygen consumption and R.Q. has been studied.

3. 2,6-DNP and 2,5-DNP in the concentrations used had no effect on the oxygen consumption or the R.Q.

4. Nembutal caused a decrease in the oxygen consumption and R.Q. of the mice.

5. Ethyl urethane or thiourea had no effect on the oxygen consumption or R.Q. of the mice.

REFERENCES

1. BONNIER, G., and TEDIN, O.: Biologisk variationsanalys. Albert Bonnier, Stockholm, 1940.
2. CLOWES, G. H. A., and KRAHL, M. E.: J. Gen. Physiol. 1938:20:145.
3. CUTTING, W. C., and TAINTER, M. L.: Proc. Soc. Exp. Biol. Med. 1932:29:1268.
4. FIELD, J. 2nd, MARTIN, A. W., and FIELD, S. M.: J. Cell. Comp. Physiol. 1934:4:405.
5. FIELD, J. 2nd, MARTIN, A. W., and FIELD, S. M.: J. Pharm. exp. Ther. 1935:53:314.
6. HALDANE, J.: J. Physiol. 1892:12:419.
7. JUDAH, J. D.: Biochem. J. 1951:49:271.
8. JUDAH, J. D., and WILLIAMS ASHMAN, H. G.: Biochem. J. 1951:48:33.
9. KENDEIGH, C. S.: J. Exp. Zool. 1939:82:419.
10. KRAHL, M. E., and CLOWES, G. A.: J. Cell. Comp. Physiol. 1938:11:1.
11. LAGERSPETZ, K., and VIILI, LEA: Ann. Med. Exp. Biol. Fenn. 1957: 35:141.
12. LOOMIS, W. F., and LIPMANN, F.: J. Biol. Chem. 1948:173:807.
13. MAGNE, H., MAYER, A., and PLANTEFOL, L.: Ann. Physiol. Physicochem. Biol. 1932:8:1.
14. MARTIN, A. W., and FIELD, J. 2nd: Proc. Soc. Exp. Biol. Med. 1937: 36:375.
15. MURALT, A. v.: Einführung in die praktische Physiologie. 1948. Springer, Berlin.
16. PARKER, V. H.: Biochem. J. 1952:51:363.
17. PEISS, C. N., and FIELD, J.: J. Biol. Chem. 1948:175:49.
18. TAINTER, M. L., BERGSTRÖM, F. W., and CUTTING, W. C.: J. Pharm. exp. Ther. 1935:53:58.
19. TOPETE, F. A.: Ciencia (México) 1951:11:301.
20. WALLGREN, H.: Acta Zool. Fenn. 1954:84:1.

EFFECT OF SOME NARCOTICS AND THIOUREA ON THE DINITROPHENOL-STIMULATED RESPIRATORY META-BOLISM OF WHITE MICE

by

KARI LAGERSPETZ and LEA VIILI

The separate effects of the substances studied here on the respiratory metabolism of the mice are presented in an earlier paper in this journal (12). The combined effect of several given substances in an organism cannot be merely predicted from the physiological effects of each substance alone. The aim of this study is to gain further insight into the action of some substances with well-known physiological effects (narcotics and thiourea) on the dinitrophenol-stimulated respiratory metabolism of mice. The discussion of the experimental results presented earlier by the authors (12) will also be included.

MATERIAL AND METHODS

The test animals, their handling, and the experimental methods were the same as described earlier (12). When the combined effects of 2,4-DNP and other substances were to be studied, the latter were injected 1.5—2 minutes after the DNP injection. At that time the external effects of DNP could already be seen. Because the narcosis caused by nembutal was only complete 15—20 minutes after the injection, the respiration measurements were not begun in these cases until this time had elapsed. In the experiments on the combined action of DNP and nembutal, DNP was injected

15 minutes after the nembutal, and measurement begun
immediately.

EXPERIMENTAL RESULTS

*The Combined Effect of 2,4-DNP and Narcotics or Thiourea
on the Respiratory Metabolism. a. Nembutal and DNP.* — As men-
tioned before, DNP was injected into the test animals 15 minutes
after the nembutal injection. In the curve for the combined action
in Fig. 1, the time interval between the two injections has been
omitted, and the time after the injections is thus counted from
the last (DNP) injection onwards. The curve for the combined
action is based on the results of nine experiments. There is no
significant difference (t = 0.26) in the oxygen consumption between
the last control values (4.84 $\pm$ 0.17) and the values obtained
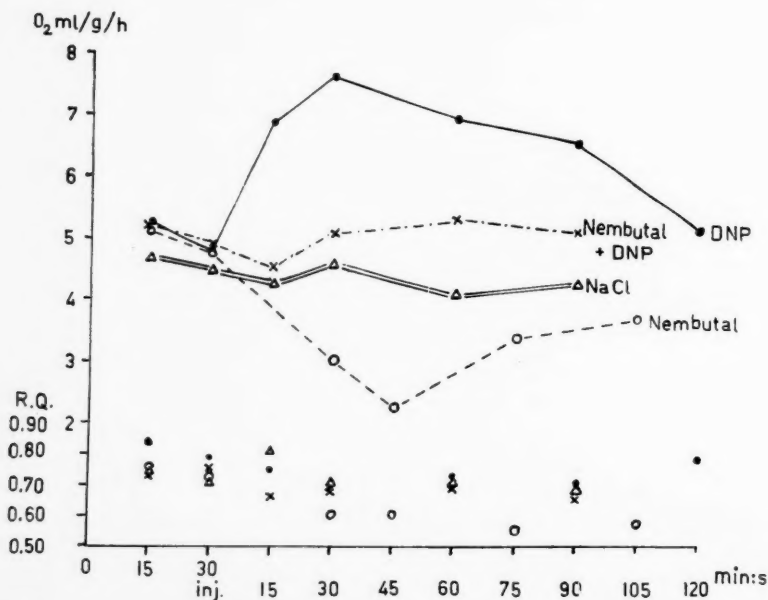
Fig. 1. — The combined action of DNP and nembutal on the oxygen consump-
tion and R.Q. Each point represents a mean of eight or nine experiments.
The values are plotted on the time scale as points at the end of each corre-
sponding measurement period. Triangles: controls with an injection of physio-
logical saline. Dots: 2 mg-% DNP injected. Circles: 50 mg/kg nembutal injected.
Crosses: 50 mg/kg nembutal injected and fifteen minutes after this 2 mg-%
DNP. The time interval between these injections is omitted. The first two
groups of points indicate the control values obtained before the injections.
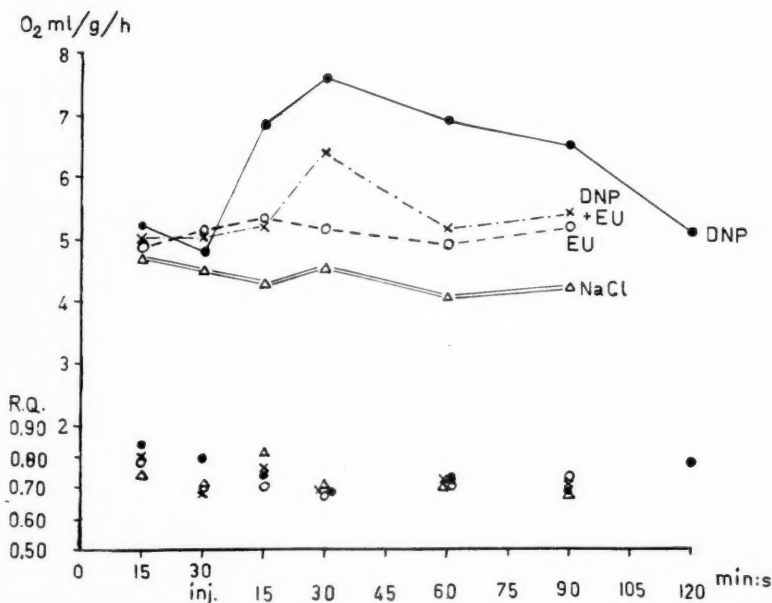
Fig. 2. — The combined action of DNP and ethyl urethane on the oxygen consumption and R.Q. Each point represents a mean of eight or nine experiments. Circles: 1 g/kg ethyl urethane injected. Crosses: 2 mg-% DNP injected, and 1.5—2 minutes after that 1 g/kg ethyl urethane. For other explanations, see Fig. 1.

during the second period of fifteen minutes after the injection (5.05 ± 0.25), during which period the stimulating effect of DNP is strongest. Although the test animals were in complete narcosis during the experiment, there was no decrease in the oxygen consumption. The decrease in R.Q. caused by nembutal was likewise eliminated if DNP was injected into animals, previously treated with nembutal. One of the test animals which received both injections died 30 minutes after the DNP injection.

DNP and Ethyl Urethane. — The behaviour of the test animals, nine in number, was as experiments with DNP alone. With this combination, there was no narcotic effect. There seems to be a statistically significant increase in the oxygen consumption (Fig. 2) (means: the second control 5.03 ± 0.29, 15—30 minutes after the injections 6.38 ± 0.29, and 75—90 minutes after the injections 5.41 ± 0.46). The value of t for the first two means is 3.46 (P = 0.01). The increase in the O_2 consumption was not so
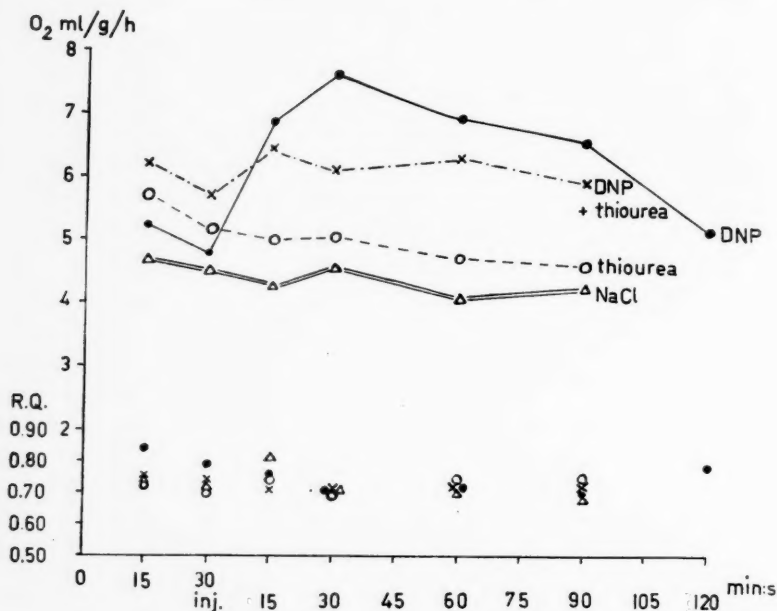
Fig. 3. — The combined action of DNP and thiourea on the oxygen consumption and R.Q. Each point represents a mean of eight or nine experiments. Circles: 1 mg-% thiourea injected. Crosses: 2 mg-% DNP injected, and 1.5—2 minutes after that 1 mg-% thiourea. For other explanations, see Fig. 1.

great as that caused by DNP alone. The R.Q. was unaffected. Although ethyl urethane alone did not influence the oxygen consumption, it obviously reduced the stimulating effect of DNP.

DNP and Thiourea. — The simultaneous injections of DNP and thiourea did not seem to affect the behaviour of the test animals. In these experiments, eight in number, there seemed to be no change in the intensity of respiration, as can be seen from Fig. 3. It must be pointed out that the oxygen consumption values in the control experiments prior to simultaneous DNP and thiourea injections lie on a higher level than the control values for the other groups in this diagram. The greatest difference in the O_2 consumption is found to exist between the means for the last control period (5.70 ± 0.66) and the first period after the injections (6.43 ± 0.39), but it is not significant ($t = 1.12$). There was no change in R.Q., either. Two of the test animals died during the experiment, although no external changes in the behaviour of the

test animals were noticed. Thiourea did not affect the normal respiration, but it obviously abolished the stimulating effect of DNP.

DISCUSSION

The first part of this study (12) comprises the results of experiments on the effect of 2,4-, 2,6-, and 2,5-dinitrophenol, nembutal, ethyl urethane, and thiourea on the oxygen consumption and R.Q.
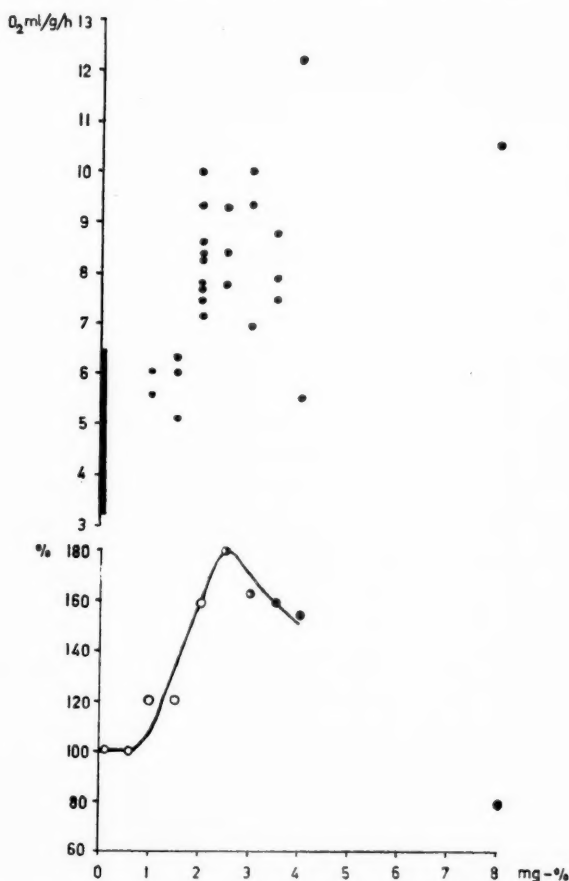
Fig. 4. — The effect of different concentrations of 2,4-DNP on the oxygen consumption. The upper diagram: the maximal individual oxygen consumption. The black rectangle at the left of the diagram represents the range of oxygen consumption of normal animals. The lower diagram: The greatest stimulation of the mean oxygen consumption during a 15-minutes period expressed as a percentage of the normal oxygen consumption (= 100%). White circles: all animals alive, half-black circles: part of animals died, black dots: all animals died durind the experiment.

of intact mice. The second part of the study was devoted to experiments on the combined action of 2,4-DNP and nembutal, 2,4-DNP and ethyl urethane, and 2,4-DNP and thiourea on the respiratory metabolism. Since intact animals were used, the differences in the activity of the animals influenced the results, and they cannot be regarded as comparable measures of the basal metabolic rate of the test animals. It was merely attempted to ascertain the differences between the effects of the various substances or concentrations used irrespectively of whether these differences depended on differences in cell respiration or, for instance, in muscular activity.

The concentration — action curve for 2,4-DNP, based on the means of the results of the experiments presented here, is given in the lower diagram of Fig. 4. It must be pointed out that in our method the readings are taken at intervals of at least fifteen minutes. Therefore, the rapid initial rise in the oxygen consumption, which is followed by a steep antemortem decrease when higher concentrations of DNP are used, does not appear from this curve. It can be seen in the upper diagram of the same figure. It is even clear in the oxygen consumption curves obtained by Tainter (15) using white rats as test animals. In his experiments, the maximal stimulation was obtained with 5 mg-% DNP, but the highest concentration which was not lethal for the rats was 3.5 mg-%, or somewhat higher than our corresponding concentration for mice. The optimal concentration for the reversible stimulation of O_2 consumption was in our experiments 2 mg-%, which nearly coincides with the optimal DNP concentration for grasshopper embryos, 1×10^{-5} M (34).

The stimulating concentrations of DNP are dependent on the temperature of the environment. Thus Fuhrman and Weymouth (11) found that 1.5 mg-% DNP only augmented the oxygen consumption of mice significantly at 30°C, but not at 25°, 20°, 15°, or 10°C. Tainter (15) could not obtain stimulation at 2—6°C, although the same concentration at 24°C markedly increased the oxygen consumption of rats. Our experiments with 1.5 mg-% DNP were performed at 19°C. Although there is a difference between the control values and the maximal stimulation value (Fig. 4), it is not significant.

In our experiments, concentrations of DNP below 1 mg-% were subliminal, the effect of concentrations of 1 and 1.5 mg-% was a

slight, but insignificant increase in O_2 consumption, and concentrations between 2 and 4 mg-% were stimulating, although all but 2 mg-% were lethal. The highest concentration used, 8 mg-%, was inhibitory. The subliminal, stimulating, and inhibitory concentrations of 2,4-DNP for the respiration of the rat brain have been reported by Fuhrman and Field (9, 10), by Peiss and Field (14), and Tyler (18), and for the respiration of rabbit lenses by Field and Tainter (8).

Bodine (1) reported an increase in the R.Q. of grasshopper embryos from 0.75 to 0.95 under the influence of DNP. This increase was also noticed in mice, although it was found to occur only when lethal concentrations (3.5 mg-% or more) of DNP were administered. The mean increase observed was from 0.73 to 0.93.

2,6-DNP and 2,5-DNP had no significant effect on the oxygen consumption or R.Q. in our experiments. Tainter, Bergström and Cutting (16) have made the same observation when working on rats and pigeons. Field, Martin and Field (7) have found that even these substances affect the respiration of yeast cells. The other effects of 2,6-DNP and 2,5-DNP found by us are previously reported in this journal (12).

Nembutal is known to possess a narcotic and respiration-inhibiting effect. In our experiments, besides these effects, there was a decrease in R.Q. from 0.73 to 0.58 (see Fig. 1 and (12)). Carbon dioxide elimination seems to be depressed more than oxygen consumption. According to our calculations, this did not occur in Topete's (17) experiments with rats. The decreases in oxygen consumption and R.Q. were abolished if 2,4-DNP was injected into the narcotized test animals, these values reaching the same levels as were measured in normal intact animals. The lowering of the respiratory metabolism caused by an anaesthetic concentration of nembutal was compensated by the action of DNP. This suggests that DNP is not inhibited by nembutal. On the other hand, Brewer (5) found that dial in urethane, in subnarcotic doses which had only a very slight inhibiting effect, if any, upon the metabolic rate of cats, totally inhibited the increase in oxygen consumption caused by DNP alone. It remains to be seen whether nembutal also in subnarcotic doses can inhibit the action of DNP.

Ethyl urethane used in a concentration of 1 g/kg body weight had no narcotic or respiration — inhibiting effect. It was able,

however, considerably to reduce the stimulating effect of DNP on oxygen consumption (Fig. 2). This, perhaps, suggests a partial antagonism between these compounds, and seems to be at variance with the results obtained by Bodine (1) with grasshopper embryos.

Although thiourea in the concentration used by us had no effect on the oxygen consumption or the R.Q. of the test animals, it suppressed the action of DNP (Fig. 3). This supports the hypothesis of Bodine (2), based on his investigations with grasshopper embryos, that DNP acts upon the thiourea — sensitive fraction of the cell respiration. It is interesting, in this connection, to notice that according to Pataky (13) thiourea, in concentrations which have no effect alone, inhibits the increase in the oxygen consumption of yeast caused by histidine, glutamic acid, glycocoll, alanine, arginine, and NH_4Cl.

The effects of the substances studied on the respiration of grasshopper embryos (Bodine and his collaborators) and mice seem generally to be rather similar. In both animals the maximal respiratory response to DNP administration is an increase in oxygen consumption of about 100%, while in many other insects, e.g. the cockroach, the increase in oxygen consumption may be 12-fold (6), or in mealworms even greater (according to unpublished experiments performed in this laboratory by Lagerspetz and Virkkula).

SUMMARY

1. The effect of some substances on the dinitrophenol-stimulated respiratory metabolism of adult white mice has been studied using the method of Haldane and Kendeigh.

2. The decrease in the oxygen consumption and R.Q. caused by nembutal was abolished by 2,4-DNP.

3. Ethyl urethane in the concentration used had no effect on the oxygen consumption of the animals, but reduced the stimulating action of 2,4-DNP.

4. Thiourea did not affect the oxygen consumption of the animals, but completely abolished the stimulating effect of 2,4-DNP.

REFERENCES

1. BODINE, J. H.: Physiol. Zoöl. 1950:*23:*63.
2. BODINE, J. H.: Physiol. Comp. Oecol. 1950:*2:*140.
3. BODINE, J. H., and BOELL, E. J.: J. Cell. Comp. Physiol. 1938: *11:*41.
4. BODINE, J. H., and FITZGERALD, L. R.: Proc. Iowa Acad. Sci. 1949: *56:*311.
5. BREWER, G.: J. Pharm. exp. Ther. 1936:*58:*135.
6. EDWARDS, G. A.: Unpublished (after ROEDER, K. et al.): Insect Physiology. 1953. Wiley & Sons, New York.
7. FIELD, J. and, MARTIN, A. W., and FIELD, S. M.: J. Pharm. exp. Ther. 1935:*53:*314.
8. FIELD, J. 2nd, and TAINTER, E. G.: Proc. Soc. Exp. Biol. Med. 1937: *36:*277.
9. FUHRMAN, F. A., and FIELD, J. 2nd: Proc. Soc. Exp. Biol. Med. 1942a:*49:*504.
10. FUHRMAN, F. A., and FIELD, J. 2nd: J. Pharm. exp. Ther. 1942b: *75:*58.
11. FUHRMAN, GERALDINE J., and WEYMOUTH, F. W.: Amer. J. Physiol. 1946:*147:*527.
12. LAGERSPETZ, K., and VIILI, LEA: Ann. Med. Exp. Biol. Fenn. 1957: *35:*131.
13. PATAKY, I.: Arch. Internat. Pharmacodyn. Thér. 1949:*78:*100.
14. PEISS, C. N., and FIELD, J.: J. Biol. Chem. 1948:*175:*49.
15. TAINTER, M. L.: J. Pharm. exp. Ther. 1934:*51:*45.
16. TAINTER, M. L., BERGSTRÖM, F. W., and CUTTING, W. C.: J. Pharm. exp. Ther. 1935:*53:*58.
17. TOPETE, F. A.: Ciencia (México) 1951:*11:*301
18. TYLER, D. B.: J. Biol. Chem. 1950:*184:*711.

FROM THE DEPARTMENT OF PATHOLOGICAL ANATOMY, UNIVERSITY OF TURKU

EFFECT OF CHLORPROMAZINE AND RESERPINE ON WOUND HEALING IN THE WHITE RAT

by

ARMO HORMIA and MARTTI HORMIA

By the use of chlorpromazine and reserpine in the treatment of psychiatric patients skin rash sometimes occurs as a complication and in some cases even trophic skin ulcerations (1, 4, 8, 9). In the light of these clinical observations alone, it is difficult to determine if it is a question only of some exceptionally reacting cases or do chlorpromazine and reserpine have a more general tendency to depress the vitality of the skin. In order to throw light upon this question the effect of parenterally applied chlorpromazine, reserpine and chlorpromazine-reserpine combination upon the wound healing was studied in the white rat.

MATERIAL AND METHODS

The investigation was performed in two parallel series. The effect of small doses of the drugs was studied by a material of 44 white male rats of the same age and the effect of large doses by a material of 44 white female rats of the same age. Both materials were divided into four subgroups, 11 rats in each. One of these acted as the control group. The three others were the chlorpromazine-, reserpine-, and combined chlorpromazine-reserpine groups. The daily dose of chlorpromazine in the series of small doses was 10 mg/kg body weight and 25 mg/kg body weight in the series of large doses. The daily doses of reserpine were 0.2 and 0.5 mg/kg

body weight respectively. The drugs were applicated subcutaneously as a single injection daily. After twentyfour hours from the beginning of the medication a wound was made by removing a round patch of skin 13 mm in diameter from the back of the rat including the skin muscle as deep as the fascia of the dorsal muscle. The epithelization of the wound was followed up daily. For grading the progress of the epithelization the times were noted when 1/4, 2/4, 3/4 and 4/4 of the area of the defect was covered by new epithelium. This estimation was made on the basis of two observers' measurings by the eye.
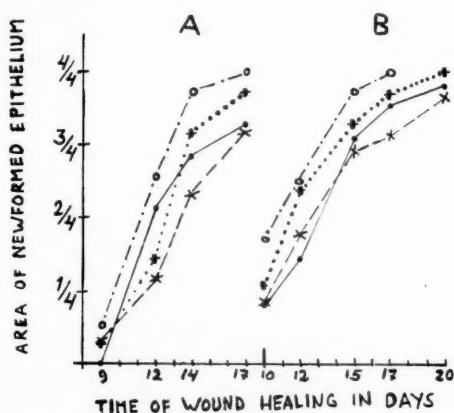
Fig. 1. — The effect of chlorpromazine ($\times$ —— $\times$ —— $\times$), reserpine ($+$ ··· $+$ ··· $+$) and combined chlorpromazine-reserpine (O— · — O · —O) upon the wound epithelization in the white rat compared with the control group ($\bullet$ — $\bullet$ — $\bullet$). A. By using small doses of the drugs (chlorpromazine 10 mg, reserpine 0,2 mg/kg body weight). B. By using large doses (chlorpromazine 25 mg, reserpine 0,5 mg/kg body weight). The figures indicate the mean area of newformed epithelium in quarters of the total surface of the wound.

RESULTS

Table 1 presents the results by using small doses of the drugs. The values show the average width of the newformed epithelium in quarters of the total area of the wound at different points. One can see from the results that the average rate of the epithelization in the chlorpromazine group was a little slower than in the control group. This retardation is, however, at no point statistically significant. In the reserpine group the epithelization is seen to be a little quickened but this, too, is not statistically significant. In the

TABLE 1

THE EFFECT OF SMALL DOSES OF CHLORPROMAZINE, RESERPINE AND COMBINED CHLORPROMAZINE-RESERPINE ON THE WOUND EPITHELIZATION IN THE WHITE RAT. THE VALUES INDICATE THE AVERAGE AREA OF THE NEWFORMED EPITHELIUM EXPRESSED IN QUARTERS FROM THE TOTAL AREA OF THE WOUND.

Time in Days from the Making of the Wound	9	12	14	17
Control group	0	2.2	2.9	3.3
Chlorpromazine (10 mg/kg) group	0.3	1.2	2.3	3.2
Reserpine (0.2 mg/kg) group................	0.3	1.5	3.2	3.7
Combined chlorpr.-reserpine group	0.5	2.6	3.7	4

group of chlorpromazine-reserpine combination, on the other hand, the epithelization is seen to be distinctly quickened. The difference to the control group is statistically significant. (The standard error of the difference between the average values of the 14th observation day = 0.28.)

TABLE 2

THE EFFECT OF LARGE DOSES OF CHLORPROMAZINE, RESERPINE AND COMBINED CHLORPROMAZINE-RESERPINE UPON THE WOUND HEALING IN THE WHITE RAT. THE TABLE IS CONSTRUCTED IN ANALOGY WITH TABLE 1.

Time in Days from the Making of the Wound	10	12	15	17	20
Control group	0.8	1.5	3.1	3.6	3.8
Chlorpromazine (25 mg/kg) group ..	0.9	1.8	2.9	3.1	3.7
Reserpine (0.5 mg/kg) group	1.1	2.4	3.3	3.7	4
Combined chlorpr.-reserpine group ..	1.7	2.5	3.7	4	

Table 2 presents the results by using large doses of the drugs. The results are similar to the results with the small doses. No statistically significant difference can be seen between the chlorpromazine and control groups. The quickening of the epithelization in the reserpine group is more evident than with small doses. The difference to the control group is statistically significant. (The standard error of the difference between the average values of the 12th observation day = 0.15.) Like in the series of small doses the epithelization is the quickest in the group of chlorpromazine-reserpine combination.

DISCUSSION

Chlorpromazine could not be shown to have any clear effect upon the rate of the epithelization of the wound. Reserpine accelerated the epithelization and this effect manifested itself clearer by using a large dosage.

The nutritional circumstances of the tissues depend decisively upon the effectiveness of their blood circulation. As is known, an insufficient circulation promotes necrotization and ulcer formation, e.g. in the skin. It has, however, not been possible to solve, if a further improvement of the circulation has any beneficial effect upon the wound healing in a previously normal tissue (6). It seems, however, to be obvious that the advantageous effect of reserpine upon the wound healing could be set in connection with its diminishing effect upon the peripheral vascular resistance and its beneficial effect upon the capillary circulation (2, 7).

The rate of the epithelization had increased most in the combined chlorpromazine-reserpine group. Consequently chlorpromazine increased the epithelization promoting effect of reserpine, although chlorpromazine alone had no beneficial effect upon the epithelization. The physiological basis of this synergistic effect of chlorpromazine and reserpine may be found in the fact that the physiological effects of these two drugs are in many respects opposed. Because of this they balance each others' disturbing effect upon the functional equilibrium of the organism (3, 10). It has been shown that even in the treatment of psychic disturbancy chlorpromazine and reserpine act mutually synergistic (5, 11). The effects of chlorpromazine and reserpine upon the wound healing described above show that the synergistic effect of these two drugs do not limit themselves solely to their psychic effects.

The results do not give support to the presumption that the skin complications sometimes seen in the psychiatric chlorpromazine and reserpine treatment should be a consequence of an impairing effect of these drugs upon the vitality of the skin.

SUMMARY

The effect of subcutaneously applicated chlorpromazine, reserpine and chlorpromazine-reserpine combination upon the wound healing was studied in the light of experiments done with two series

of 44 white rats. Chlorpromazine was used in daily doses of 10 mg and 25 mg/kg body weight, reserpine in doses of 0.2 and 0.5 mg/kg body weight. Chlorpromazine could not be shown essentially to influence the epithelization. Reserpine shortened it somewhat. Chlorpromazine-reserpine combination accelerated the wound healing clearly. Chlorpromazine and reserpine thus have a mutually synergistic effect upon the rate of the wound healing.

REFERENCES

1. ANTON-STEPHENS, D.: J. ment. Sc. 1954:*100:*543.
2. AZIMA, H., KENT, J., and CLEGHORN, R.: Canad. Med. Ass. J. 1955: *73:*866.
3. BARSA, J., and KLINE, N.: Arch. Neur. Psychiat. 1955:*74:*280.
4. DENBER, H.: J.A.M.A. 1955:*158:*586.
5. EIBER, H.: Arch. Neur. Psychiat. 1955:*74:*36.
6. FORBUS, W.: Reaction to Injury. — Baltimore 1952.
7. HAFKENSCHIEL, J., SELLERS, A., KING, G., and THORNER, M.: Ann. N.Y. Acad. Sc. 1955:*61:*78.
8. HORMIA, A.: Duodecim 1956:*72:*76.
9. KASAK, M., SCHAEFER, D., and WINTER, W.: Marquette Med. Rev. 1955:*21:*17.
10. KINROSS-WRIGHT, V.: Ann. N. Y. Acad. Sc. 1955:*61:*174.
11. LEMERE, F.: J. Neur. Psychiat. 1955:*74:*1.

STUDIES OF SERUM LIPIDS AND LIPOPROTEINS IN BLEEDING ANAEMIA IN RATS

by

MATTI MIETTINEN

Acute bleeding anaemia in rabbits causes a pronounced rise in the amounts of serum cholesterol, phospholipids and total lipids (*e.g.*, 2, 15). The cholesterol and phospholipids are generally increased in the beta lipoprotein fraction only and reduced in most cases in the alpha lipoprotein fraction, so that the percentages of cholesterol and phospholipids in alpha lipoprotein are definitely decreased (6).

Bloor (1) reported that the serum cholesterol is not increased in bleeding anaemia in the dog. Blood letting from the chicken in an amount corresponding to one-third of its blood volume resulted in six hours to a fall in the serum cholesterol. After 30 hours the cholesterol had regained normal value, and one week after bleeding the cholesterol was above normal (10).

Youngburg and Youngburg (19) bled from four rats 4—5 ml. of blood on every third day during 15—20 days and determined the serum phospholipids in each blood sample. No definite changes were observed by them in the amount of phospholipids, although on the average they were slightly reduced. They published no data on possible changes in the amounts of haemoglobin and red cells.

The above mentioned studies seem to indicate that bleeding anaemia in the dog does not produce changes in the serum lipids similar to those produced in the rabbit. In the dog about 90 per cent of the serum cholesterol and phospholipids are bound in alpha$_1$

lipoprotein (11), while in the rabbit about 34 per cent of the choles-
terol and about 50 per cent of the phospholipids are in alpha$_1$ lipo-
protein (7). In the rat about 65 per cent of the serum cholesterol
and phospholipids is in the alpha$_1$ lipoprotein fraction, about 20
per cent in the alpha$_2$ lipoprotein fraction, and about 15 per cent
in the beta lipoprotein fraction (8). These studies as well as investi-
gations on, *e.g.*, experimental atherosclerosis demonstrate differ-
ences in fat metabolism of the dog, rat and rabbit. Since the
present writer did not find in the literature any investigations on
the effect of bleeding anaemia on the serum lipids and lipoproteins
of the rat, the performance of the present study was considered
desirable.

MATERIAL AND METHODS

The series comprised 24 white rats, weight 200—300 gr. From
the tail of three rats 2—3 ml. of blood was let daily; for the lipo-
protein analyses 4—5 ml. was let from these rats. From 21 rats the
daily amount taken was 4—7 ml. The blood was best obtained under
a rapid ether narcosis, so that the rat did not react to the cutting of
the tail between the vertebrae with a sharp knife. Administration of
the narcotic was discontinued shortly before the required volume
of blood had been bled and the rat usually revived from the ana-
esthesia immediately after the blood letting. The effort was to
induce as rapid and superficial anaesthesia as possible but one
which prevented the rat from reacting to cutting of the tail and
from moving during bleeding. The haemoglobin value was deter-
mined according to Sahli from every sample, and the results are
reported as corrected Sahli values.

The serum cholesterol and phospholipids were determined by the
method presented by Nikkilä (9) and the total lipids by the method
of Swahn (16). The distribution of the cholesterol and phospho-
lipids in the lipoprotein fractions in paper electrophoresis was deter-
mined in the manner described in another paper by the present
writer (8).

Analyses of the cholesterol, phospholipids and total lipids
were made from each blood sample. The distribution of the phos-
pholipids in the lipoprotein fractions was determined from the
first blood sample from 24 rats and thereafter only from occasional

samples, usually when the haemoglobin value was lowest. The distribution of cholesterol in the lipoprotein fractions was determined for 13 rats from the same blood samples that were used for determination of phospholipid distribution.

RESULTS

Table 1 and fig. 1 show the mean values of analyses on three rats on which preliminary experiments were made to study effect of bleeding anaemia on the serum lipids and lipoproteins. In the first round the rats were bleed about 5 ml. of blood, from which the serum cholesterol, phospholipids, total lipids and distribution of cholesterol and phospholipids in the lipoprotein fractions were studied. From strips of cut from the paper after the electrophoretic run and stained with Sudan black to localize the lipoprotein fractions it was seen that the greater part of the lipoproteins had migrated to the albumin fraction position. At alpha$_2$ and beta globulin positions there were weak lipoprotein fractions, of which the latter adjoined without a distinct margin the lipids absorbed into the paper. It was decided to analyse the alpha$_2$ and beta lipoprotein fractions together because of their small amount.

After the first sample each rat was bled daily 2—3 ml. of blood from which the serum cholesterol, phospholipids, total lipids and haemoglobin were analysed. On the sixth day about 5 ml. was bled and also the lipoproteins were analysed as described above. It was observed that the amounts of cholesterol and phospholipids in the alpha$_2$ + beta lipoprotein were slightly increased. On the eighth day 3—5 ml. was taken from each rat and since the amount of haemoglobin had a increasing trend the same amount was bled also on the ninth and tenth days. Lipoprotein determinations were also made from these samples. The strips of paper stained with Sudan black for localisation of the lipoproteins revealed a marked increase in the alpha$_2$ lipoprotein. The beta lipoproteins were also increased, whereas the lipoproteins at the albumin fraction had decreased. In accordance with this observation, the cholesterol and phospholipids at the albumin fraction were determined thereafter in the same manner as from the earlier samples but the alpha$_2$ and beta lipoprotein fractions were cut apart and the cholesterol and phospholipids were determined separately from each of these frac-
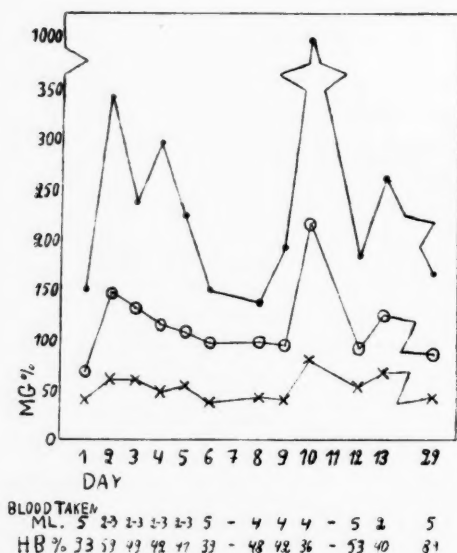
Fig. 1. — Mean values for serum total lipids, phospholipids and cholesterol in the serum of three rats bled 2—5 ml daily.
Under the graph are listed the volumes bled daily and the mean daily haemoglobin values.

● = total lipids;   ⊙ = phospholipids;   × = cholesterol

TABLE 1

MEAN CHANGES IN SERUM LIPID AND LIPOPROTEIN VALUES IN BLEEDING ANAEMIA IN THREE RATS. HAEMOGLOBIN VALUES STUDIED ON THE SAME DAYS ARE PRESENTED IN FIG. 1

Lipid	Day	Total mg %	Alpha$_1$ %	Alpha$_2$ %	Beta %	Alpha$_1$ mg %	Alpha$_2$ mg %	Beta mg %
Phospholipids	1	69	72	28		50	19	
»	6	97	52	48		50	47	
»	10	215	10	63	27	20	138	57
»	29	85	65	20	15	55	17	14
Cholesterol	1	43	64	36		26*	14	
»	6	38	47	53		18	20	
»	10	80	12	59	29	8	48	24

tions. The lipids which were absorbed into the paper at origin were analysed together with the beta lipoprotein fraction, since no distinct margin was present between them. As is seen from table 1, the cholesterol and phospholipids in the lipoproteins at the albumin position (which are termed alpha$_1$ lipoprotein) were decreased both absolutely and relatively, while the cholesterol and phospholipids in alpha$_2$, and to a lesser extent, those in beta lipoprotein, were absolutely and relatively icreased.

The following blood sample (5—6 ml.) was taken on the twelfth day from the beginning of bleeding period. The haemoglobin was now definitely increased and the serum cholesterol, phospholipids and total lipids had declined to normal levels. On the following day these lipids were again slightly elevated. The last blood sample from these rats was taken on the 29th day. The anaemia was now cured and the serum cholesterol, phospholipids and total lipids were at normal levels. The relative and absolute values of phospholipids in the alpha$_1$, alpha$_2$ and beta lipoprotein fractions were now also normal.

On the basis of the results from these preliminary experiments it was decided to bleed 4—7 ml. daily from the following rats. From each sample, determinations were made of the haemoglobin and the serum cholesterol, phospholipids and total lipids. The distribution of cholesterol and phospholipids in the alpha$_1$, alpha$_2$ and beta lipoprotein fractions was determined from the first sample and was repeated a second time 2—4 days after the beginning of the bleeding period, when the serum began to show opalescence or turbidity because of lipaemia. For some rats these lipoproteins were determined more frequently than from two samples. The cholesterol in the lipoprotein fractions was determined for 10 rats and the phospholipids for 21 rats. The results of these determinations are shown in table 2 and fig. 2.

It will be seen from the results that acute bleeding anaemia in rats produced a statistically significant rise in the amounts of serum cholesterol, phospholipids and total lipids. The increase was relatively greater in the total lipids than in cholesterol and phospholipids. The phospholipids, again, increased more than cholesterol, which is evident from the statistically significant rise in the phospholipi d/cholesterol ratio.

The increase in cholesterol and phospholipids occurred chiefly
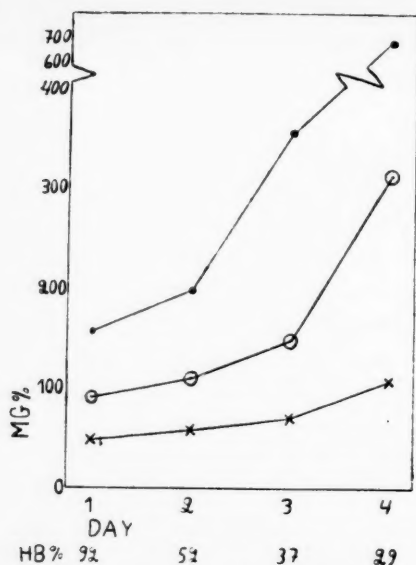
Fig. 2. — Mean values for serum total lipids, phospholipids and cholesterol
in the serum of 18 rats bled 4—7 ml. daily on four days.
Numbers below the graph are the mean daily haemoglobin values.

● = total lipids;   ⊙ = phospholipids;   × = cholesterol

TABLE 2

MEAN VALUES FOR SERUM LIPIDS AND LIPOPROTEINS IN THE FIRST SAMPLE AND
THE MEAN CHANGES IN 21 RATS BLED 4—7 ML DAILY

Lipid			No. of Rats	Mean from First Samples	Range		Mean Change	Standard Error	t	P
					Min.	Max.				
Phospholipids,	total	mg %	21	92	58	170	+132	20	6.6	0.001
»	alpha$_1$	%	21	66.7	59.0	75.1	− 40.7	2.4	17.0	0.001
»	alpha$_2$	%	21	20.0	15.1	27.9	+ 38.0	2.4	15.8	0.001
»	beta	%	21	13.3	8.5	19.4	+ 2.8	1.0	2.8	0.02
»	alpha$_1$ mg %		21	61	39	110	− 11	4.0	2.8	0.02
»	alpha$_2$ mg %		21	19	11	35	+119	16	7.4	0.001
»	beta	mg %	21	12	7	25	+ 24	3.6	6.7	0.001
Cholesterol,	total	mg %	21	50	32	76	+ 52	9.0	5.8	0.001
»	alpha$_1$	%	10	62.0	52.6	71.4	− 38	5.0	7.6	0.001
»	alpha$_2$	%	10	23.7	14.3	31.7	+ 35	5.0	7.0	0.001
»	beta	%	10	14.4	11.4	17.8	+ 2.8	0.6	4.7	0.002
»	alpha$_1$ mg %		10	35	23	50	− 12	3.8	3.2	0.02
»	alpha$_2$ mg %		10	13	7	20	+ 62	14	4.4	0.002
»	beta	mg %	10	8	5	13	+ 12	2.6	4.6	0.002
P : C ratio,	total		21	1.83	1.34	2.27	+ 0.35	0.07	5.0	0.001
»	alpha$_1$		10	2.04	1.82	2.34	+ 0.41	0.20	2.0	..
»	alpha$_2$		10	1.69	1.40	2.33	+ 0.54	0.16	3.4	0.01
»	beta		10	1.44	1.00	1.92	+ 0.52	0.16	3.3	0.01
Total lipids		mg %	21	153	78	278	+505	75	6.7	0.001

in the alpha$_2$lipoprotein fraction, both the absolute and relative values of the cholesterol and phospholipids in this fraction increasing significantly. The absolute and relative values of cholesterol and phospholipids in the beta lipoprotein fraction also increased significantly. The ratio between phospholipids and cholesterol in the alpha$_2$ and beta lipoprotein fractions calculated from the absolute amounts of these lipids showed a significant increase. The percentages of cholesterol and phospholipids in alpha$_1$ lipoprotein decreased significantly. Also the corresponding absolute amounts showed a slight decline, the fall being statistically almost significant.

For four rats the distribution of phospholipids in the alpha$_1$, alpha$_2$ and beta lipoprotein fractions was determined also from the second blood sample. The changes in the lipoproteins showed a similar trend as those seen in the later samples, although they were considerably less marked with the exception that the absolute amount of phospholipids in alpha$_1$ lipoprotein had not declined and in one case was, on the contrary, definitely increased. In two cases the distribution of cholesterol and phospholipids was determined 4—5 days after the beginning of the bleeding period. In these samples the absolute values for cholesterol and phospholipids in alpha$_1$ lipoprotein were considerably higher than those in the sample taken on the third day and in the first sample.

DISCUSSION

As shown by the presented results, acute bleeding anaemia in rats produced marked changes in the serum lipids and lipoproteins. The total lipids increased relatively more than the cholesterol and phospholipids. This points to a possibility that the greatest increase occurred in neutral fats. There was a significantly greater increase in the phospholipids than in the cholesterol. The increase in phospholipids and cholesterol occurred chiefly in the alpha$_2$ lipoprotein fraction. The amounts of these lipids in the beta lipoprotein fraction also increased significantly. On the other hand, the absolute values of these lipids in the alpha$_1$ lipoprotein fraction declined in most cases, the fall being statistically almost significant about three days after commencement of the bleeding period. In two cases the distribution of cholesterol and phospholipids in the lipo-

protein fractions was determined also 4—5 days after commencement of bleeding when the absolute amounts of cholesterol and phospholipids in alpha$_1$ lipoprotein were considerably higher than in the sample taken 3 days after commencement and higher than in the first sample. These findings seem to indicate the possibility that the decrease in cholesterol and phospholipids in the alpha$_1$ lipoprotein fraction 3 days after beginning of bleeding may possibly change to a rise on the following days.

When the rats were bled daily 2—3 ml. it appeared to produce no considerable changes in the serum lipids and lipoproteins. Only after the volume bled was increased to about 4 ml. the above mentioned changes occurred in the serum lipids and lipoproteins. The serum cholesterol, phospholipids and total lipids declined to normal levels within two days in the three rats in this series in which bleeding was omitted on one day. During these two days the haemoglobin increased, on an average, from 36 per cent to 53 per cent. When bleeding 4 rats 4—5 ml. on every third day during 15—20 days Youngburg and Youngburg (19) observed no definite changes in the serum phospholipids. The amount of blood taken may have been too small to produce sufficiently rapidly a anaemia of such severity as to cause a rise in the serum phospholipids.

It has been reported that the administration of cortisone to rats raises the serum cholesterol (*e.g.*, 17). It also may be mentioned that cortisone increases chylomicronaemia in rats due to fat ingestion (3). Acute stress is also stated to slightly raise the serum cholesterol in man (4) and serum fat in rat (18). Prolonged exposure to cold elevates total lipids and cholesterol in rat serum (13). Since an acute haemorrhage and anoxia are stresses (14) and since anoxia produces an increase of adrenal function (12), it seems probable that the stress due to acute haemorrhage may be a possible cause of the increased serum cholesterol, phospholipids and total lipids in bleeding anaemia in rats. The writer has in other connection expressed the opinion that stress may be a possible reason for the increase seen in the above mentioned lipids in acute haemorrhagic anaemias in the rabbit also (6).

It has been suggested that liver lesion due to anaemic anoxia may be the cause of the low cholesterol and phospholipid amounts in alpha$_1$ lipoprotein in bleeding anaemias in rabbits (6). The thought is near at hand that the same factor may be the cause of

the low absolute cholesterol and phospholipid values in alpha$_1$ lipoprotein in acute bleeding anaemias also in rats. An interesting difference in the serum lipoprotein changes in bleeding anaemia in the rat and the rabbit is that in the rat the increase in cholesterol and phospholipids occurs chiefly in the alpha$_2$ lipoprotein traction, whereas in the rabbit it is seen in the beta lipoprotein (6). In the present study with rats the increase was significantly greater in the phospholipids than in the cholesterol, whereas in rabbits the increase was approximately as great in both the phospholipids and cholesterol (6). In haemorrhagic anaemia in man the values for serum cholesterol, phospholipids and total lipids and the absolute and relative values for cholesterol and phospholipids in alpha lipoproteins and the absolute amount of cholesterol in beta lipoprotein were significantly below normal (5).

SUMMARY

A study using 24 rats was made of the effect of bleeding anaemia on the amount of serum cholesterol, phospholipids and total lipids and on the distribution of cholesterol and phospholipids in alpha$_1$, alpha$_2$ and beta lipoproteins fractionated by paper electrophoresis. When 4—7 ml. of blood was bled daily from the rat tail, lipaemic turbidity appeared in the serum 2—4 days after the beginning of the bleeding period and the serum cholesterol, phospholipids and total lipids showed a statistically significant increase. The rise was relatively greatest in the total lipids, and phospholipids increased significantly more than cholesterol. The increase in cholesterol and phospholipids occurred chiefly in the alpha$_2$ lipoprotein fraction, but also in the beta lipoprotein fraction these lipids were significantly increased. The absolute amounts of cholesterol and phospholipids in the alpha$_1$ lipoprotein fraction decreased slightly in most cases, as a result of which the percentages of these lipids in alpha$_1$ lipoprotein showed a marked fall.

BIBLIOGRAPHY

1. BLOOR, W. R.: J. Biol. Chem. 1921:49:201.
2. BOGGS, T. R., and MORRIS, R. S.: J. Exp. Med. 1909:11:553.
3. HAKKILA, R. L., and HALONEN, P. I.: Ann. med. exp. biol. Fenn. 1953:31:36.
4. KUHL, W. J., Jr., WILSON, H., and RALLI, E. P.: J. Clin. Endocrinol. Metab. 1952:12:393.
5. MIETTINEN, M.: Ann. med. int. Fenn. 1956:45:suppl. 22.
6. MIETTINEN, M.: Ann. med exp. biol. Fenn. 1957:35:Suppl. 10.
7. MIETTINEN, M.: Ann. med. exp. biol. Fenn. 1957:35:165.
8. MIETTINEN, M.: Ann. med. exp. biol. Fenn. 1957:35:168.
9. NIKKILÄ, E.: Scand. J. Clin. Lab. Invest. 1953:5:Suppl. 8.
10. RODBARD, S.: Circulation 1955:12:498.
11. RUSS, E. M., and RAYMUNT, J.: Circ. Res. 1955:3:194.
12. SCHNEPPENHEIM, P., and HUHN, A.: Beitr. pathol. Anat. 1955:115:117.
13. SELLERS, E. A., and ROSEMARY, W. Y.: Brit. Med. J. 1956:4971:815.
14. SELYE, H.: Stress, Montreal 1950.
15. STARUP, U.: Undersøgelser over experimentel Hyperlipaemi. Munksgaard. København. 1937.
16. SWAHN, B.: Scand. J. Clin. Lab. Invest. 1953:5:Suppl. 9.
17. WINTER, C. A., SILBER, R. H., and STOERK, H. C.: Endocrinology 1950:47:60.
18. YANKOVLEV, N. N.: Fiziol. Z. 1952:38:332. Abstract in Excerpta med., sec. II. 1953:6:907.
19. YOUNGBURG, G. E., and YOUNGBURG, M. V.: J. Lab. Clin. Med. 1936: 21:798.

DISTRIBUTION OF CHOLESTEROL AND PHOSPHOLIPIDS IN RABBIT SERUM LIPOPROTEIN FRACTIONS SEPARATED BY PAPER ELECTROPHORESIS

by

MATTI MIETTINEN

Of the serum lipids in the rabbit about 30 per cent are bound in alpha lipoprotein when the lipids are fractioned by paper electrophoresis and stained with oil red 0 (2) or with Sudan black (*e.g.*, 1). Lewis *et al.* (5) studied the serum lipoproteins of the rabbit by ultracentrifugation and found that the concentration of $alpha_1$ lipoprotein ($S_{1.21}$ 1—15) was about 100 mg per 100 cc and that of beta lipoprotein about 50 mg per 100 cc. Havel *et al.* (4) combined preparative ultracentrifugation with chemical analysis of the separated fractions and investigated the distribution of cholesterol and phospholipids in the lipoprotein fractions of one rabbit. In this rabbit 41 per cent of the cholesterol and 74 per cent of the phospholipids were bound in low density ($D < 1.063$) lipoproteins.

Since the writer has been unable to find in the literature any studies on the distribution of cholesterol and phospholipids in the lipoprotein fractions of rabbit serum separated by paper electrophoresis it seems desirable to report the results of a study made on this subject.

MATERIAL AND METHODS

The amounts of total serum lipids, cholesterol and phospholipids and the distribution of the cholesterol and phospholipids in paper

electrophoretically separated alpha and beta lipoproteins were analysed in 39 rabbits.

The total serum lipids were analysed by the method of Swahn (9) and the cholesterol and phospholipids by the method described by Nikkilä (8).

The serum lipoproteins were separated by Nikkilä's (8) method as modified by Miettinen (6). The cholesterol and phospholipids in alpha and beta lipoproteins were analysed in the same manner as these lipoprotein fractions in human serum were analysed in an earlier study (6), with the exception that 0.8 ml serum was pipetted for each filter paper sheet, while 0.25 ml was enough for human serum.

RESULTS AND COMMENTS

Table 1 shows the results of the serum lipid and lipoprotein analyses. It will be observed that about 34 per cent of the cholesterol and about 50 per cent of the phospholipids were bound in the alpha lipoprotein. The amounts of cholesterol and phospholipids in high density lipoproteins analysed in one rabbit by preparative, ultracentrifugation by Havel *et al.* (4) lay within the range of the values in alpha lipoprotein in the present study. The percentage of total lipids (sudanophilic material) in alpha lipoprotein was about 30 per cent in a study by Antonini *et al.* (1) and thus was

TABLE 1

SERUM LIPIDS AND LIPOPROTEINS IN 39 RABBITS

Lipid		Mean	Range		Standard Deviation	Standard Error
			Min.	Max.		
Phospholipids,	total, mg %	84	47	150	24	3.8
»	alpha, %	50	22	73	13	2.1
»	alpha, mg %	42	14	79	15	2.4
»	beta, mg %	42	19	102	18	2.8
Cholesterol,	total, mg %	45	15	131	22	3.6
»	alpha, %	34	10	63	13	2.1
»	alpha, mg %	14	2	27	5	0.7
»	beta, mg %	31	10	117	20	3.2
P : C ratio,	total	2.10	0.91	3.71	0.65	0.11
»	alpha	3.23	1.26	7.00	1.17	0.19
»	beta	1.57	0.73	3.47	0.53	0.09
Total lipids	......mg %	194	69	383	74	11.9

lower than the percentage of cholesterol and phospholipids in alpha lipoprotein in the present work. The reason for this is probably the relatively lower content of neutral fats in alpha than in beta lipoprotein (*e.g.*, 9, 3).

In normal human serum about 29 per cent of the cholesterol and 49 per cent of the phospholipids are bound in alpha lipoprotein (6) when analysed by the method used in this investigation. In rabbits the distribution of cholesterol and phospholipids in the lipoprotein fractions is nearly the same as in humans. In the present study the amounts of cholesterol and phospholipids migrating between $alpha_1$ and beta lipoproteins ($= alpha_2$ lipoprotein) were determined in a few cases. The percentage of these lipids was about 2—10 per cent, which is about the same as in humans (6). In rats the relative and absolute amounts of cholesterol and phospholipids in $alpha_1$ and $alpha_2$ lipoproteins are higher than in rabbits and the amounts in the beta lipoprotein are very low (7).

SUMMARY

The distribution of serum cholesterol and phospholipids in alpha and beta lipoprotein fractions separated by paper electrophoresis was studied in 39 white rabbits. About 34 per cent of the total amount of cholesterol and about 50 per cent of the phospholipids were bound to alpha lipoprotein. The phospholipid/cholesterol ratio was 3.2 in the alpha lipoprotein fraction and 1.6 in the beta fraction.

BIBLIOGRAPHY

1. ANTONINI, F. M., GRANDONICO, E. and PIVA, G.: Sperimentale 1953: *103:*331.
2. BOSSAK, E. T., WANG, CH.-I., and ADLERSBERG, D.: Proc. Soc. Exp. Biol. & Med. 1954:*87:*637.
3. GOFMAN, J. W., DE LALLA, O., GLAZIER, F., FREEMAN, N. K., LINDGREN, F. T., NICHOLS, A. V., STRISOWER, B., and TAMPLIN, A. R.: Plasma 1954:*2:*413.
4. HAVEL, R. J., EDER, H. A., and BRAGDON, J. H.: J. Clin. Invest. 1955: *34:*1345.
5. LEWIS, L. A., GREEN, A. A., and PAGE, I. H.: Am. J. Physiol. 1952: *171:*391.
6. MIETTINEN, M.: Ann. med. int. Fenn. 1956:*45:*Suppl. 22.
7. MIETTINEN, M.: Ann. med. exp. biol. Fenn. 1957:*35:*168.
8. NIKKILÄ, E.: Scand. J. Clin. Lab. Invest. 1953:*5:*Suppl. 8.
9. SWAHN, B.: Scand. J. Clin. Lab. Invest. 1953:*5:*Suppl. 9.

DISTRIBUTION OF CHOLESTEROL AND PHOSPHOLIPIDS IN RAT SERUM LIPOPROTEIN FRACTIONS SEPARATED BY PAPER ELECTROPHORESIS

by

MATTI MIETTINEN

The greater proportion of the lipids in rat serum are in the form of alpha$_1$ lipoproteins (2). In studying rat serum lipoproteins by ultracentrifugation Lewis *et al.* (2) observed that alpha$_1$ lipoproteins (S$_{1.21}$ 1—15) were present in amounts of about 100 mg per 100 cc and beta$_1$ lipoproteins (S$_{1.21}$ 25—40) in amounts of about 30 mg per 100 cc. Normally there were no lipoproteins of lower density in the rat. Havel *et al.* (1) combined preparative ultracentrifugation with chemical analysis of the separated fractions and analysed by this method the distribution of the serum cholesterol and phospholipids in the lipoprotein fractions in one rat. In this rat 61 per cent of the cholesterol and 68 per cent of the phospholipids were bound in high density (D $>$ 1.063) lipoproteins.

Since the writer has been unable to find in the literature any studies on the distribution of cholesterol and phospholipids in the lipoprotein fractions of rat serum separated by paper electrophoresis it has seemed desirable to report the results of a study made on this subject.

MATERIAL AND METHODS

The amounts of total serum lipids, cholesterol and phospholipids, and the distribution of the phospholipids in the alpha$_1$, alpha$_2$ and beta lipoprotein fractions were analysed in 21 rats. The distri-

bution of the cholesterol in these fractions was studied in 10 rats. The animals were white rats weighing 200—300 gr.

The fractionation of the serum lipoproteins was based on the method fo Nikkilä (7) so modified as to adapt it to the study of serum lipoproteins in the rat. Whatman filter paper No. 1 was cut into sheets 24×30 cm in size and 0.5 ml of serum was pipetted on each sheet. After the electrophoretic run a strip 1 cm wide was cut from the margin of the paper and the proteins in the strip were stained with azocarmine by the method of Turba and Enenkel (8). In another strip 2 cm wide the lipoproteins were stained with Sudan black B according to Swahn (9). These stainings demonstrated the most distinct lipoprotein fraction at the albumin level (alpha$_1$ lipoprotein), and a slight and indefinite one at the beta globulin. Since the lipoproteins which had become absorbed at the origin and those which had migrated to the beta globulin position could not reliably be separated and since they were very small in amount, they were regarded as one fraction (= beta lipoprotein). There was a rather faintly distinguisable lipoprotein fraction between alpha$_1$ and beta lipoproteins (= alpha$_2$ lipoprotein), which, however, was more distinct than that in the beta globulin position.

When the paper from which the strips had been cut for the above described protein and lipoprotein stainings was viewed against daylight, the alpha$_2$ and beta lipoprotein fractions were found to run in a straight line parallel to the starting line. In ultraviolet light the albumin band was clearly discernible by its fluorescence and was slightly curved at the margins of the paper. On basis of the fluorescence it was possible to determine where the albumin fraction had migrated farthest from origin. At a distance of about 3 mm from this point towards the anode end a line was drawn to designate the margin of the alpha$_1$ lipoprotein fraction on the anode side. The boundary between alpha$_1$ and alpha$_2$ lipoproteins was drawn along the margin of the albumin fraction on the cathode side. The margin of the beta globulin on the anode side formed the boundary between alpha$_2$ and beta lipoproteins. The cathode boundary of the beta lipoprotein was drawn at a distance of 12 mm on the cathode side of the starting line. The lipids were extracted from the three strips cut along these boundaries, containing the alpha$_1$, alpha$_2$ and beta lipoprotein fractions. The phospholipids were analysed from one strip and the cholesterol from another strip

according to the method of Nikkilä (7) in a slightly modified form (3). Points which should be taken into consideration in calculating the results have been discussed earlier by the present author (3). The cholesterol and phospholipid amounts in the lipoprotein fractions in the rat were calculated in the same manner as for man and rabbit, with the exception that a blank test value of 0.005 was subtracted from the cholesterol extinction in each lipoprotein fraction, whereas for man and rabbit this value is 0.01. The reason for this is the smaller size of the paper containing each lipoprotein fraction of the rat and therefore the lower blank test value for the rat.

The serum cholesterol and phospholipids were determined according to Nikkilä (7) and the total lipids according to Swahn (9).

RESULTS

Table 1 shows the results of serum lipid and lipoprotein analyses for 21 rats. It will be observed that most of the cholesterol and

TABLE 1

SERUM LIPIDS AND LIPOPROTEINS IN RATS

Lipid			No. of Rats	Mean	Range		Standard Devia-tion	Standard Error
					Min.	Max.		
Phospholipids,	total	mg %	21	92	58	170	23.0	5.0
»	alpha₁	%	»	67	59	75	3.7	0.8
»	alpha₂	%	»	20	15	28	3.4	0.7
»	beta	%	»	13	9	19	3.3	0.7
»	alpha₁	mg %	»	61	39	110	16.7	3.7
»	alpha₂	mg %	»	19	11	35	6.0	1.3
»	beta	mg %	»	12	7	25	3.7	0.8
Cholesterol,	total	mg %	»	50	32	75	12.9	2.8
»	alpha₁	%	10	62	53	71	6.1	1.9
»	alpha₂	%	»	24	14	32	5.7	1.8
»	beta	%	»	14	11	18	2.1	0.7
»	alpha₁	mg %	»	35	23	50	8.8	2.8
»	alpha₂	mg %	»	13	7	20	4.2	1.3
»	beta	mg %	»	8	5	13	2.6	0.8
P: C ratio,	total		21	1.83	1.34	2.27	0.20	0.04
»	alpha₁		10	2.04	1.82	2.34	0.17	0.05
»	alpha₂		»	1.69	1.40	2.33	0.30	0.10
»	beta		»	1.44	1.00	1.92	0.30	0.10
Total lipids		mg %	21	153	78	278	56.6	12.4

phospholipids in rat resum were in the form of alpha$_1$ lipoprotein. The amounts of cholesterol and phospholipids in alpha$_2$ lipoprotein were slightly higher than the amounts of these lipids in the beta lipoprotein fraction. The phospholipid/cholesterol ratio was highest in the alpha$_1$ lipoprotein fraction, and slightly higher in the alpha$_2$ fraction than in the beta fraction.

DISCUSSION

According to the results presented, about 60—70 per cent of the cholesterol and phospholipids in rat serum were bound to alpha$_1$ lipoprotein. This result is in agreement with those obtained by Havel *et al.* (1) by the ultracentrifuge method. About 21 per cent of the cholesterol and phospholipids were present in the alpha$_2$ lipoprotein fraction and about 14 per cent in the beta fraction. Using the same method as in the present study, it has been found that in the serum of healthy persons about 28 per cent of the cholesterol and about 49 per cent of the phospholipids were bound in alpha$_1$ lipoproteins (3). For rabbit serum these figures were about 34 per cent and about 50 per cent, respectively (4). About 2—8 per cent of the cholesterol and phospholipids in human and rabbit serum were in the form of alpha$_2$ lipoproteins, according to the same studies. Thus in the rat both the absolute and relative values of cholesterol and phospholipids in beta lipoprotein were very low as compared to the values in rabbit and especially in man. On the other hand, the absolute and relative values of cholesterol and phospholipids in alpha$_1$ and alpha$_2$ lipoproteins were higher in the rat than in the rabbit. In this connection it may be pointed out as a feature of some interest that in acute haemorrhagic anaemias in the rat the amount of serum cholesterol and phospholipids is increased greatly in the alpha$_2$ lipoprotein fraction and to some extent also in the beta fraction (6), whereas in the rabbit the increase in these lipids occurred in the beta lipoprotein fraction (5). The relative and absolute amounts of cholesterol and phospholipids in alpha$_1$ lipoprotein were usually reduced in haemorrhagic anaemias in both the rabbit and the rat.

SUMMARY

The distribution of serum phospholipids and cholesterol in alpha$_1$, alpha$_2$ and beta lipoproteins fractionated by paper electrophoresis was studied in rats, using 21 animals for the phospholipid and 10 rats for the cholesterol analyses. Of the total amounts of phospholipids and cholesterol, about 60—70 per cent was bound in the alpha$_1$ fraction, about 21 per cent in the alpha$_2$ fraction and about 14 per cent in the beta fraction. The ratio of the absolute amounts of phospholipids and cholesterol was 2.04 in the alpha$_1$ lipoprotein fraction, 1.69 in the alpha$_2$ fraction, and 1.44 in the beta fraction.

BIBLIOGRAPHY

1. HAVEL, R. J., EDER, H. A., and BRAGDON, J. H.: J. Clin. Investig. 1955:*34:*1345.
2. LEVIS, L. A., GREEN, A. A., and PAGE, I. H.: Am. J. Physiol. 1952: *171:*391.
3. MIETTINEN, M.: Ann. med. int. Fenn. 1956:*45:*Suppl. 22.
4. MIETTINEN, M.: Ann. med. exp. biol. Fenn. 1957:*35:*165.
5. MIETTINEN, M.: Ann. med. exp. biol. Fenn. 1957:Suppl. 10.
6. MIETTINEN, M.: Ann. med. exp. biol. Fenn. 1957:*35:*155.
7. NIKKILÄ, E.: Scand. J. Clin. Lab. Invest. 1953:*5:*Suppl. 8.
8. TURBA, F., and ENENKEL, H. J.: Naturwissenschaft 1950:*37:*93.
9. SWAHN, B.: Scand. J. Clin. Lab. Invest. 1953:*5:*Suppl. 9.

OBSERVATIONS ON THE DETERMINATION OF GLUCURONIC ACID

by

T. MIETTINEN, V. RYHÄNEN and H. SALOMAA

No fully specific method for the determination of uronic acids has been presented so far. The most widely used methods are Tollens's naphthoresorcinol reaction (20, 21, 22), in which the colour obtained with hydrochloric acid and naphthoresorcinol is extracted with ether, and Dische's carbazol reaction (2), in which the colour after strong acid hydrolysis is obtained with carbazol. Modifications of Tollens's reaction differ from each other mainly in regard to the substance used in the extraction. In addition to ether (1, 11, 12, 15, 16) there have been used for this purpose toluene (4), amyl alcohol (8) and butyl acetate (6).

In the following we have used a method in which the extraction is performed with butyl acetate. This substance was chosen because, in spite of the strong fluorescence, it gives a crystal clear violet colour and the addition of alcohol is not necessary in the extraction.

We have not wanted to use ether because of its inflammability, evaporation, and formation of peroxides. With toluene on the other hand, we sometimes have had turbidities, which very often were visible in side light only, thus interfering the photoelectrical measurement. Furthermore already very small amounts of protein prevent extraction of the colour.

PROCEDURE

Reagents: 1) Concentrated HCl (Merck pro anal.). — 2) Naphtho-resorcinol solution (0.2%): 200 mg of naphthoresorcinol (Merck pro anal.) in 100 ml of water is shaken for 10 min. in the machine, then kept for 24 h. at 37°C. After cooling and filtering the solution is ready for use. A fresh solution is prepared daily. — 3) Butyl acetate (85%) normal (Merck pro anal.). — 4) Sodium tungstate 10% (Merck pro anal.). — 5) Sulfuric acid 2/3-n (Merck pro anal.).

Into the tubes with glass stoppers are pipetted 2 ml of the fluid to be analysed and 4 ml of a mixture containing 2 parts of naphthoresorcinol solution and 2 parts of concentrated HCl. This mixture is prepared just before pipetting. The tubes are closed, shaken and placed for 30 min. in the water bath at 98°C, after which they are cooled in ice water and 4 ml of butyl acetate is pipetted into each tube. When shaken for 30 sec. a strongly fluorescent layer of a crystal clear violet colour is formed. The water layer is removed by suction from the bottom of the tubes and the photoelectrical measurement of the colour layer is performed with a Beckman B photometre, using wavelength 570 mμ. The photoelectrical measurement was performed against water except in the case of absorption curves, when it was performed against the blank. Since the standard curve, usually made with 5 γ—30 γ amounts of glucurone (Fig. 1), has a tendency to change, there was included in every series a control tube containing 10 γ of glucurone. As standard solution was used a glucurone solution containing 1 mg/100 ml and kept in the ice box.

The proteins were removed by precipitation with 10% sodium tungstate and 2/3-n sulfuric acid. This was performed as follows:

1) Plasma, 1 ml; water, 8 ml; sodium tungstate, $\frac{1}{2}$ ml; sulf. acid, $\frac{1}{2}$ ml.

2) Blood, 1 ml; water 7 ml; sodium tungstate, 1 ml; sulf. acid, 1 ml.

3) Unwashed red cells centrifugated for about 10 min, 1 ml; water, 11 ml; sodium tungstate 1$\frac{1}{2}$ ml; sulf. acid, 1$\frac{1}{2}$ ml.

After standing for about 5 min. the mixture is centrifugated. The dilution of the protein-free supernatant is 1:10 for plasma and serum and 1:15 for red cells. After the precipitation the pH must be about 2.5.

From the solutions obtained in this way are taken 2 ml and 1 ml (water *ad* 2 ml) and the determination is performed as presented above. Precipitations sometimes arising during the hydrolysis originate most frequently from an incomplete precipitation of proteins.

The urine was diluted 1:50, 1:100, 1:200, a.s.o., according to its concentration, so that 2 ml of the dilution did not contain more than 30 γ of glucuronic acid.[1]

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[1] All naphthoresorcinol-positive substances are expressed here as glucuronic acid and correspond quantitatively to glucurone.

RESULTS

Glucurone, galacturonic, acid hyaluronic acid and chondroitin-sulfuric acid have only one peak in the absorption curve, at 570 mμ (Fig. 2). Glucose, galactose and mannose have the peak at 510 mμ. Plasma, blood, red cells, urine and combination of glucose and glucurone have peaks at both points (Fig. 1 and 2). Galacturonic acid reacts more strongly than the same amount of glucurone as appears already from figures 1 and 2.
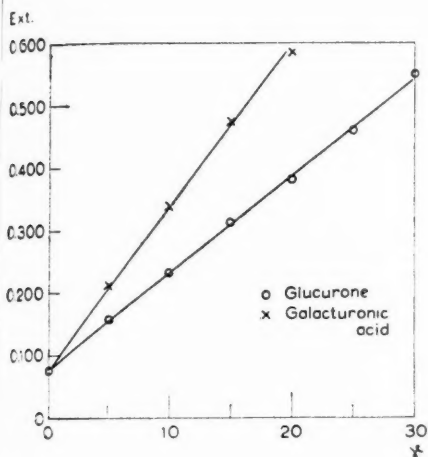
Fig. 1. — Standard curves for glucurone and galacturonic acid measured at 570 mμ.
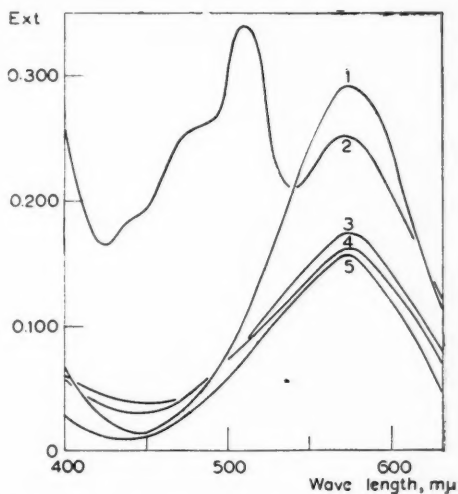
Fig. 2. — Absorption curves for: 1. Galacturonic acid, 10 γ; 2. Urine; 3. Hyaluronic acid, 200 γ; 4. Chondroitin sulf., 200 γ; 5. Glucurone, 10 γ.

The glucuronic acid values obtained from plasma, blood and urine are presented in tables 1 and 2. In red cells there was an average of 8.7 mg%. After addition of glucurone to plasma, blood and urine the values ranged from 89.5% to 108.5% of the calculated values (Table 3). A definite proportionality is seen between the values obtained with the separate dilutions (Table 4).

About 10% of the total glucuronic acid content of hyaluronic acid was normally obtained.
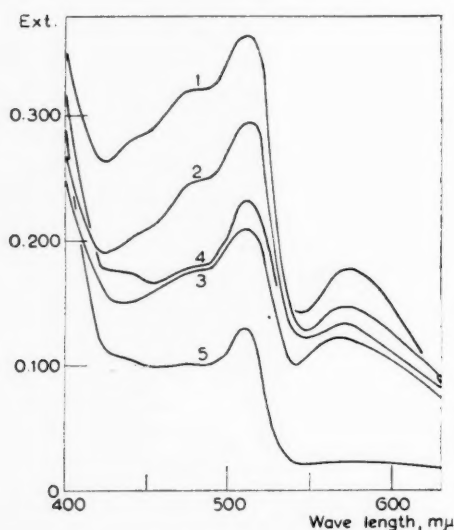
Fig. 3. — Absorption curves for: 1. Red cells; 2. Blood; 3. Plasma; 4. Glucose, 200 γ and glucurone. 10 γ; 5. Glucose, 200 γ.

TABLE 1

AMOUNTS OF GLUCURONIC ACID IN HUMAN PLASMA AND BLOOD

Person No.	1	2	3	4	5	6	7	8	Mean
Plasma mg %	2.5	3.3	3.3	2.7	3.5	3.5	2.9	3.1	3.1
Blood »	4.5	6.8	6.4	6.4	6.5	6.7	5.0	5.7	6.1

TABLE 2

EXCRETION OF GLUCURONIC ACID INTO URINE DURING THREE CONSECUTIVE DAYS

Subject		Mg/1st Day	Mg/2nd Day	Mg/3rd Day	Mean
Person	No. 1	502.4	530.2	590.5	541.0
»	2	542.3	559.0	465.0	522.1
»	3	285.2	491.5	350.4	375.8
»	4	477.3	762.4	741.0	660.2
»	5	298.6	318.0	279.0	298.6
Guinea pig No. 1		17.5	16.2	22.9	18.9
	2	27.5	16.6	25.4	23.2
	3	44.8	39.0	33.6	39.2
Rat	No. 1	5.7	6.9	4.5	5.7
	2	6.6	4.7	2.7	4.7

TABLE 3

RECOVERY OF GLUCURONE ADDED INTO PLASMA AND BLOOD (5 γ) AND INTO URINE (10 γ). THE ADDITION INTO PLASMA AND BLOOD WAS MADE BEFORE THE PRECIPITATION OF PROTEINS

Substance	Obtained Values (γ)	Calcutaled Values (γ)	Obtained Values in % of Calculated
Blood	8.5	9.5	89.5
»	9.8	9.5	103.2
»	11.5	11.8	97.5
Plasma	7.4	8.2	90.5
»	8.2	8.2	100.0
»	9.5	8.8	108.0
»	11.7	11.2	104.5
Urine	21.8	21.8	100.0
»	30.5	28.5	107.0
»	30.7	28.5	108.5
»	24.5	22.5	108.9
»	14.8	15.4	96.7
»	15.0	15.4	97.5

TABLE 4

PROPORTIONALITY OF RESULTS OBTAINED WITH VARIOUS DILUTIONS

Dilution	Plasma (γ)			Blood (γ)			Dilution	Urine (γ)		
1 : 10	6.7	6.5	5.4	10.5	12.3	14.1	1 : 50	23.3	12.3	24.2
1 : 20	3.4	3.2	2.8	5.0	6.4	6.8	1 : 100	11.3	5.8	12.0
1 : 40	..	1.8	..	2.6	2.9	3.3	1 : 200	5.6	3.0	6.3

COMPARISON OF THE RESULTS

The first peak in the absorption curves is caused by interfering substances and it thus depicts their amount. Thus red cells appear to contain relatively large amounts of such substances. The absorption curves fig. 3 differs markedly from those obtained by some other methods (4, 10, 12, 17, e.c.) In all of the latter, however, the peak of the glucuronic acid is at 560—580 mμ.

The glucuronic acid values here obtained, *i.e.*, 2.5—3.5 mg% for plasma and 4.6—6.8 mg% for blood, correspond on the whole to the average of those reported in the literature, which is 0.4— 6.0 mg% for plasma (1, 3, 5, 6, 7, 10, 18) and 4.0—9.2 mg% for

blood (4, 10, 16). The red cells have been calculated to contain 73% of the glucuronic acid in the blood, which would correspond to 7.7 mg% (10). Values calculated in the same way would here be 72% and 9.6 mg%.

The normal excretion of glucuronic acid into human urine has been stated to be 230—1325 mg in 24 h., depending on the method used (3, 4, 5, 7, 8, 10, 12, 19, 22). Most of these values fall between 400 and 500 mg in 24 h. The corresponding value for the guinea pig is 28 mg (14) and for different strains of rats 1.1 and 17.7 mg (13). The results obtained here correspond well with those described above.

The 10% of the total glucuronic acid content of hyaluronic acid obtained in the present study exceeds markedly that reported by Hollman (9), which was 3.4%.

SUMMARY

Some observations have been presented concerning the determination of uronic acids based on the naphthoresorcinol reaction modified by Mozolowski (15). Butyl acetate was used to extract the colour produced by HCl and naphthoresorcinol. The photoelectrical measurement was performed at wavelength 570 mμ. The standard curve was prepared with glucurone. The proteins were precipitated by the sodium tungstate-sulfuric acid method.

The glucuronic acid values were 3.1 mg% for plasma and 6.0 mg% for blood. Into the urine of man, guinea pig and rat there was excreted in 24 h. the averages of 477.9 mg, 27.1 and 5.2 mg of glucuronic acid, respectively. These results are compared with those reported in the literature.

In the absorption curves prepared with different substances peaks occur at 510 mμ and 570 mμ if interfering substances are present. Uronic acid on the other hand, have the peak at 570 mμ only. The photometre readings at the mentioned points depict the relationship between the interfering substances and uronic acids.

REFERENCES

1. DEICHMAN, W., and DIERKER, M. J.: J. Biol. Chem. 1946:*163*:753.
2. DISCHE, Z. J.: J. Biol. Chem. 1947:*167*:189.
3. DOWBEN, R. M.: J. Clin. Investigation 1956:*35*:277.
4. FISHMAN, W. H., SMITH, M., THOMPSON, D. B., BONNER, C. D., KASDON, C. D., and HOMBURGER, F.: J. Clin. Investigation 1951:*30*:685.
5. FISHMAN, W. H., and GREEN, S. : J. Biol. Chem. 1955:*215*:527.
6. DE FRATES, J. S., and BOYD, M. J.: Federation Proc. 1953:*12*:194.
7. FREEMAN, S., KANABROCKI, E. L., and INMAN, C. W.: J. Lab. & Clin. Med. 1956:*47*:583.
8. HANSON, S. W. F., HILLS, G. T., and WILLIAMS, R. T.: Biochem. J. 1944:*38*:274.
9. HOLLMAN, S.: Zschr. f. physiol. Chem. 1954:*298*:77.
10. HOLLMAN, S., and WILLE, E.: Zschr. f. physiol. Chem. 1952:*290*:91.
11. KAPP, E. M.: J. Biol. Chem. 1940:*134*:143.
12. MAUGHAN, G. B., EVELYN, A., and BROWNE, J. S. L.: J. Biol. Chem. 1938:*126*:567.
13. MOSBACH, E. H., JACKEL, S. S., and KING, C. G.: Arch. Biochem. 1950:*29*:348.
14. MOSBACH, E. H., and KING, C. G.: J. Biol. Chem. 1950:*185*:491.
15. MOZOLOWSKI, W.: Biochem. J. 1940:*34*, 2: 823.
16. RATTISH, H. D., and BULLOWA, J. G. M.: Arch. Biochem. 1943:*2*:381.
17. STARY, Z., and YUVANIDIS, M.: Biochem. Zschr. 1953:*324*:206.
18. SUDHOF, H.: Klin. Wchnschr. 1952:*30*:86.
19. SUDHOF, H., and SCHELLONG, G.: Klin. Wchnschr. 1953:*31*:64.
20. TOLLENS, B.: Ber. dtsch. chem. Ges. 1908:*41*:1788.
21. TOLLENS, C.: Zschr. f. physiol. Chem. 1908:*56*:115.
22. TOLLENS, C.: Zschr. f. physiol. Chem. 1909:*61*:95.

ULTRAVIOLET IRRADIATION OF BLOOD

EFFECT ON PLASMA POTENTIAL AND THE OXIDATION REDUCTION CAPACITY, »POISING EFFECT» OF PLASMA

by

EEVA JALAVISTO[1] and J. SUNDBERG

Irradiation of blood with ultraviolet light in vitro, and in vivo has been studied primarily for development of methods for sterilization of blood (»Knott Technic»). Many profound effects on the various elements of blood have been noted (1). Most of them concern the viability and reactions of leucocytes. More general aspects are considered in a paper by Giani (4), who noted differences in the oxidation reduction processes, *e.g.* a reduction in the concentration of reduced glutathione and a corresponding increase in the GSS form present in whole blood after irradiation. Staffe and Darguzas (12) report on inactivation of catalase by ultraviolet irradiation of haemolyzed rabbit and guinea pig blood. Seyderhelm (13, 14) has found that injections of UV-irradiated blood samples oppose the anemizing effect of saponin by enhancing the output of erythrocytes from bone marrow in the recipient animals. The same effect is seen when blood is irradiated when streaming in vivo.

Very few studies concern the mechanism of the action of UV-rays on blood, but the number of investigations dealing with various pure systems is considerable. *E.g.* it has been shown that various enzymes are inactivated by UV-irradiation. The proteins

show both oxidative and nonoxidative denaturation (3) whereby liberation of sulfhydryl groups may occur (8, 9, 10). Furthermore, ovalbumin irradiated with UV-light inactivates added insulin (9). The studies indicate that UV-irradiation interferes with oxidation reduction phenomena in the medium subjected to irradiation. Since erythropoietic stimulation by repeated bleeding (6, 11) and by low pressure (5) is connected with changes in the electrode potential (»oxidation reduction potential»), it deemed worth while to examine the effect of irradiation with UV-light on the plasma potential and the oxidation reduction capacity (»poising effect») of plasma in order to gain some insight on factors possibly connected with the effects of ultraviolet irradiation of blood on erythropoiesis.

MATERIAL AND METHODS

Samples of blood were obtained from 15 rabbits partly by heart puncture partly, in 6 rabbits, from carotid loops, and from 12 dogs by venipuncture. The experimental procedure was as follows. The blood sample was divided in two or three portions. One blood sample was irradiated in quartz cuvettes of 1 cm thickness (Beckman spectrophotometer cuvettes) at a distance of 20 cm from an ordinary therapeutic Hg-arch lamp during 1 hour, and mixed thoroughly after $\frac{1}{2}$ hours' irradiation. The second portion was kept in ordinary light in room temperature and served as control. Both samples were centrifuged immediately thereafter and the plasma separated from the cells. The third portion was centrifuged and the plasma irradiated as was the first sample.

In every instance the initial plasma potential a, was determined after 10 minutes' stabilization time as described in a previous paper (5). A control for purity of the electrode by taking the potential reading of the electrode in an unpoised phosphate buffer solution of pH 7.4 preceded each determination. The electrode was cleaned, rinsed with water, dryied and glewed until the potential amounted to at least 250 mV. At approximately 12 min. after introduction of the plasma sample in the Luer syringe which was fitted with a platinum plate electrode (reference electrode: saturated calomel electrode) 0.2 ml of a 35 mg per cent aqueous solution of ascorbic acid was added by suction to 1 ml plasma in the syringe.

The potential was read anew at 8 minutes after addition of ascorbic acid and 10 min. after the first reading. The difference in potential, Δ brought forth by addition of ascorbic acid was taken to represent the »poising capacity» of blood plasma. In some later experiments the electrometric titration of the plasma with ascorbic acid was continued until the minimum potential, β, obtainable with successive additions of ascorbic acid, was reached or surpassed. The »poising capacity», γ, was then calculated by dividing the range of potential, $a-\beta$, by Δ. The determination of γ was made in 7 experiments with dog blood.

For comparison some experiments with irradiation of pure human 19 per cent albumin solution[1] were performed.

RESULTS

When blood is irradiated, the potential of plasma increases quite regularly. As seen from table 1, which summarizes the results obtained with dog blood, the difference in a, 23.3 ± 6.31 mV, is statistically significant at the P = 0.001 level. The potential drop, Δ, effected by addition of 0.2 ml of ascorbic acid to the plasma sample was likewise definitely greater in plasma separated from irradiated blood UV_B, the difference being 22.0 ± 3.12 mV (P < 0.001). In the 7 cases when γ was measured it was uniformly decreased. Evidently the poising capacity tends to be lowered both if judged by the drop in potential, Δ, or when the shift in initial potential and the whole range, $a-\beta$, is taken into account by calculation of γ.

In five instances plasma was irradiated. The results were quite consistent: the initial potential was approximately the same as in the plasma separated from irradiated blood but the drop in potential effected by addition of ascorbic acid, Δ, was much greater. The difference between the Δ of irradiated plasma and control was nearly twice the difference between Δ of plasma separated from irradiated blood and control Δ.

The irradiation experiments performed with rabbit blood, gave similar results as those with dog blood (table 2). The initial potential a of plasma and the potential drop, Δ, are increased after irradiation

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[1] It is a pleasure to a acknowledge my indébtedness to Dr. E. Uroma, Chief of the State Serum Institute, for furnishing the albumin solution.

TABLE 1

Date	Dog	a			Δ			γ [2]		
		C[1]	UV_B	UV_P	C	UV_B	UV_P	C	UV_B	Diff.
3. 5. 54	H	11	98	—	26	77	—	—	—	—
24. 5. 54	H	25	40	49	35	44	87	—	—	—
6. 5. 54	P	62	66	—	48	49	—	—	—	—
2. 6. 54	P	38	82	88	42	81	89	—	—	—
3. 5. 54	A	36	90	—	46	66	—	—	—	—
24. 5. 54	A	25	50	44	41	65	94	—	—	—
4. 5. 54	M	80	61	—	61	61	—	—	—	—
25. 5. 54	M	31	44	40	35	69	85	—	—	—
4. 5. 54	T	58	60	—	49	57	—	—	—	—
25. 5. 54	T	13	43	42	37	55	84	—	—	—
12. 1. 55	T	33	82	—	37	81	—	1.40	1.21	—0.19
18. 8. 55	T	80	$\begin{cases}88\\90\end{cases}$	—	60	$\begin{cases}72\\74\end{cases}$	—	1.50	$\begin{cases}1.30\\1.27\end{cases}$	$\begin{cases}-0.20\\-0.23\end{cases}$
30. 4. 54	Pl	55	75	—	46	75	—	—	—	—
4. 5. 54	L	65	75	—	55	74	—	—	—	—
6. 5. 54	K	80	69	—	52	60	—	—	—	—
2. 6. 54	V	32	60	—	46	61	—	—	—	—
10. 1. 55	V	56	75	—	53	79	—	1.36	1.22	—0.14
12. 1. 55	V	69	110	—	49	89	—	1.41	1.22	—0.19
3. 5. 54	F	30	75	—	51	70	—	—	—	—
25. 5. 54	F	29	25	—	48	62	—	—	—	—
11. 1. 55	F	48	81	—	48	71	—	1.40	1.28	—0.12
30. 8. 55	B	53	72	—	41	70	—	1.24	1.20	—0.04
»	Bo	58	83	—	58	80	—	1.48	1.26	—0.22
		46.4 ± 4.8	69.7 ± 4.1	52.6 ± 8.3	46.2 ± 2.0	68.2 ± 2.4	88.0 ± 1.8	1.40	1.26	—0.16
		Diff. 23.3 ± 6.31			Diff. 22.0 ± 3.12					
		$t = 3.69$			$t = 7.0$					
		$P < 0.001$			$P < 0.001$					

[1] $=$ Control. [2] $\gamma = \dfrac{\text{range of potential}}{\Delta} = \dfrac{a - \beta}{\Delta}$

of blood. The picture is, however, quite changed when rabbit blood plasma is irradiated without the presence of red cells. The plasma potential is now decreased on an average by 49.5 ± 11.1 m which is highly significant ($P < 0.001$). The change in potential caused by addition of ascorbic acid does not show any significant difference when compared with the control sample, but is significantly less than in plasma samples separated from the irradiated blood. Because of the enormous negativation of the potential of irradiated

TABLE 2

Date	Rabbit	a			Δ		
		Control	UV-Irr. Blood	UV-Irr. Plasma	Control	UV-Irr. Blood	UV-Irr. Plasma
1954							
11. 6.	265	50	63	— 75	41	55	— 5
»	286	56	70	— 23	58	66	20
»	238	50	49	—110	50	48	—22
»	239	22	39	— 10	47	53	34
7. 5.	III art.	12	36	— 44	22	31	19
9. 6.	»	32	59	— 30	34	51	26
7. 5.	Rus. art.	11	32	— 80	24	34	6
5. 6.	»	20	35	— 27	39	42	26
8. 6.	»	5	23	50	28	36	50
9. 6.	215 art.	34	63	— 9	45	63	33
»	Hop.	12	20	± 0	25	31	37
»	Lem. art.	29	32	— 10	33	40	32
5. 6.	420	46	55	1	42	56	46
8. 6.	»	26	48	55	37	49	67
5. 6.	II art.	24	32	— 20	26	35	40
8. 6.	»	20	39	17	35	44	50
15. 5.	MEph.	35	39	— 43	25	26	—11
		28.5 ± 3.7	43.1 ± 3.7	-21 ± 9.3	36.0 ± 2.6	44.7 ± 3.0	26.3 ± 5.83
		Diff.: 14.6 ± 5.23		64.1 ± 10.0	8.7 ± 3.89		18.4 ± 6.53
		$t = 2.79$		$t = 6.41$	$t = 2.24$		$t = 2.82$
		$p < 0.01$		$p < 0.001$	$p < 0.05$		$p < 0.01$

Δ is $+$ when addition of ascorbic acid lowers the potential

Δ is — when addition of ascorbic acid results in an increase (positivation) of the potential.

plasma the shift in potential affer addition of ascorbic acid is sometimes negative *i.e.* in stead of a further negativation as always in the control samples, an increase of the potential is seen. This is, of course, what one would expect if assumed that ascorbic acid tends to establish a certain definite potential level in plasma. It is therefore more remarkable, that these cases are so few. In 11 cases the initial potential a, is less than the value in the control after addition of ascorbic acid, but nevertheless a further negativation occurs in 8 out of them.

Some experiments with irradiation of a 19 percent albumin solution yielded results which resemble those obtained with irradiation of plasma, only in exaggerated form. The shift in

potential caused by irradiation is enormous, amounting sometimes
to 235—350 mV. The addition of ascorbic acid leads now to a
positivation of the potential the positivation being regularly less
than the negativation of the control sample (0.2—0.7 $\times$ Δ control).
(Table 3) If the poising effect of a solution is defined as the capacity

TABLE 3
HUMAN ALBUMIN SOLUTION, 19 PER CENT

| Date | a | | Δ | | Difference | |
| | | | | | a | Δ |
	Control	Irradiated	Control	Irradiated	Irr.—Control	Irr.—Control
11. 5. 54	152	—198	124	—88	—350	—212
17. 5. 54	132	—103	80	—40	—235	—120
18. 5. 54	130	—179	92	—60	—309	—152
26. 5. 54	158	— 68	117[1]	—38[1]	—226	—155

Δ is $+$ when addition of reducing solution decreases the electrode potential.
Δ is — when addition of reducing substance results in an increase (positivation) of the potential.

[1] Reducing solution: 0.1 ml of a 0.0001 per cent solution of adrenaline.

of it to resist to the effect on electrode potential of an addition of
some reducing on oxidizing agent, the irradiation with UV-light
has increased the poising effect of the albumin solution. In order
to make sure that there is no question of a specific reaction to
ascorbic acid, adrenalin was used as reducing agent in stead of
ascorbic acid in one experiment. The effect was the same, although
the conformity of absolute values must be considered purely con-

TABLE 4

| Plasma Sample | 25. 2. 54 | | 26. 2. 54 | | 2. 3. 54 | |
	a	Δ	a	Δ	a	Δ
Control	35[1]	35[1]	40	43	24	26
30 % hemol. plasma[2]	—	—	35	45	—	—
50 % hemolyzed plasma	28	31	—	—	—	—
100 % hemolyzed plasma[2]	22	25	48	45	30	27

[1] 10 per cent aq. dest. added to the plasma.
[2] hemolyzed plasma, 100%, obtained by addition of 1 part aq. dest. to
9 parts blood.
The 30 or 50 per cent samples are obtained by diluting 3 or 5 parts of
(100%) hemolyzed plasma with 7 or 5 parts of control plasma.

incidental. By varying the amount of adrenalin added to the solution different values for Δ naturally will be obtained.

Since hemolysis of the irradiated blood sample was not avoidable some experiments on the effect of hemolysis on the poising effect were performed. The hemolysis was effected by addition of 1 part distelled water to 9 parts of the sample. A nonhemolytic control plasma sample was then diluted with this hemolytic plasma. The results appear in table 4. As may be seen the effect of hemolysis on Δ is either none or opposite to the effect of irradiation of blood and can consequently not be responsible for the differences found between irradiated and unirradiated samples.

<div align="center">DISCUSSION</div>

The mechanism of the positivation of blood plasma potential after irradiation of blood by UV-light, cannot be asessed on basis of this study. Several possibilities may however, be considered. The fact that irradiation of whole blood and plasma yielded quite different results (positivation of the potential, when blood was irradiated and negativation, when plasma samples were irradiated) probably depends partly upon the absence of catalase in plasma samples and its presence in red cells which prevents the formation of H_2O_2 in the blood sample. Because of absorption of UV-light by the red cell pigment the penetration of the UV radiation in deeper layers of the sample is not possible. A weaker effect in the irradiated blood than in plasma is therefore to be expected, but a reversal of the shift in potential *i.e.* a qualitatively different effect can hardly result from a more superficial action.

The experiments show an interspecies difference in plasma potential a which is highly significant, viz. 17.9 ± 6.06 mV; $t = 2.95$ and $P < 0.01$. A corresponding difference in the value of Δ is likewise manifested. The difference: $\Delta_{dog} - \Delta_{rabbit} = 10.2 \pm 3.28$ mV is significant at the $P = 0.005$ level t being 3.11. According to the definition of »poising capacity» adopted in this paper, this would mean that dog blood plasma is more weakly »poised» than that of rabbit blood. Whether this is a true species difference or only conditioned by *e.g.* dietary differences remains unsettled. However, it shows that the method of recording the

electrode potential may be considered sensitive enough to studies of this kind and that it seems to yield reproducible values.

If the effect of irradiation of blood is compared with the effects of hypoxic exposure or bleeding on the electrode potential it may be noted that the shift in potential is of comparable magnitude and of same direction. Whether these facts and the observations of Seyderhelm suggesting that blood becomes erythropoietically active when irradiated, have some common denominator or are directly interrelated phenomena cannot be settled on basis of these experiments.

As long as the fundamental stimulus of blood formation is not known, the oxidation reduction processes as manifested in changes of plasma potential, deserve further study.

SUMMARY

The effect of irradiation with ultraviolet light of dog and rabbit blood and plasma on the oxidation reduction properties of plasma was studied by electrometric determinations of the plasma potential before and after addition of 0.2 ml of an 0.035 per cent aqueous solution of ascorbic acid to 1 ml of plasma.

A significant interspecies difference in the plasma potential a, and in the shift of potential caused by the addition of ascorbic acid Δ was noted. The potential a in dog plasma was 46.4 ± 4.8 and that in rabbit plasma 28.5 ± 3.7. The shift in potential Δ, was likewise greater in dog plasma which thus appears to be more weakly poised than rabbit plasma ($\Delta_{dog} = 46.2 \pm 2.0$ and $\Delta_{rabbit} = 36.0 \pm 2.6$). The irradiation with ultraviolet light of blood increased the plasma electrode potential a and the shift in potential Δ, a by 23.3 ± 6.31 in dog and 14.6 ± 5.23 in rabbit and Δ, respectively, by 22.0 ± 3.1 in dog and 8.7 ± 3.9 in rabbit plasma.

When rabbit plasma was irradiated without the presence of red cells and particularly if human albumin solutions were irradiated a very strong drop in electrode potential was noted. Δ was also uniformely decreased, which is interpreted as a manifestation of an increase in the poising capacity of plasma and of albumin solutions after irradiation.

REFERENCES

1. BARGER, G. J. P., and KNOTT, E. K.: in Glasser, O, Medical Physics II, 1950.
2. BEMBÉ, C., and DIETRICH, S.: Z. exper. Med. 1941:*109*:546.
3. FIOLA, S.: Bioch. Z. 1947:*318*:67.
4. GIANI, E.: Bull. Soc. ital. biol. sper. 1947:*23*:357.
5. JALAVISTO, E.: Ann. med. exp. Fenn. 1954:*32*:178.
6. JALAVISTO, E., and PIHA, S.: Ann. med. exp. Fenn. 1952:*30*:361.
7. JALAVISTO, E., and SUNDBERG, J.: Acta Physiol. Scand. 1954:*31*: suppl. 114.26.
8. KATHER, E.: Arch. exp. Path. Pharm. 1937:*184*:645.
9. KATHER, E.: Ibid. 1937:*185*:323.
10. MIRSKY, A. E., and ANSON, M. L.: J. gen. Physiol. 1936:*19*:427.
11. PIHA, S.: Studies on Blood Regeneration during Progressive Reticulo-cytosis Due to Repeated Bleedings. Helsinki, 1956.
12. STAFFE, A., and DARGUZAS, V.: Acta haemat. 1951:*5*:36.
13. SEYDERHELM, R.: Klin. Wschr. 1932:*11*:628.
14. SEYDERHELM, R., and KREITMAIR: Arch. exper. Path. Pharm. 1932: *167*:106.

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ÜBER DEN EINFLUSS VON N_1-SULFANILYL-N_2-n-BUTYL-CARBAMID AUF DIE EXPERIMENTELLE ATHERO-SKLEROSE BEI HÄHNCHEN

von

SVEN PUNSAR und GOTTFRIED HÄRTEL

Seit Einführung des Insulins in die Therapie des Diabetes mellitus bilden die Arteriosklerose und ihre Folgezustände die Haupttodesursache bei dieser Krankheit. Nach Joslin (11) sterben heutzutage 69,1% der Diabetiker infolge einer Gefässkrankheit und nur 1,9% infolge des diabetischen Komas. Wie aus dem von Liebow und Hellerstein (15) zusammengestellten Material hervorgeht, tritt Koronarsklerose bei Diabetikern häufiger auf als bei anderen Personen gleichen Alters — nach Heinsen (9) sogar 4—5mal häufiger als bei Stoffwechselgesunden — und Katz (12) ist der Ansicht, dass die Verhütung der degenerativen Gefässerkrankungen zur Zeit das Hauptproblem der Diabetesbehandlung darstellt.

Die Arterioskleroseneigung des Diabetikers wird in letzter Zeit mehr für eine Folge von Störungen des Gesamtstoffwechsels gehalten als für eine reine Fettstoffwechselstörung (19). Welche Rolle dem Insulin dabei zukommt, ist jedoch unklar. Root und Wilson (18) vermuten einen direkten Zusammenhang zwischen dem Ausmass der Diabeteskontrolle und den Faktoren, die zu vorzeitigem Auftreten von atherosklerotischen Gefässveränderungen führen. Schettler (19) behauptet, dass Gefässschäden bei gut eingestellten Diabetikern nicht häufiger aufträten als bei Nichtdiabetikern. Beides spricht dafür, dass Insulinmangel und Entgleisen

des Stoffwechsels ein vermehrtes Auftreten von Atherosklerose zu Folge haben. Im Gegensatz zu den obigen klinischen Feststellungen haben Versuche von Stamler, Pick und Katz jedoch ergeben, dass zugeführtes Insulin bei normalen Hähnchen die Regression experimentell hervorgerufener Koronarsklerose verhindert bzw. verzögert (22) und dass gleichzeitige Verabfolgung von cholesterinhaltigem Futter und Insulin stärkere atherosklerotische Veränderungen an Aorta und Koronargefässen verursacht als cholesterinhaltiges Futter allein (23).

Seit rund einem Jahr wird in mehreren europäischen Ländern ein orales Antidiabeticum — N_1-sulfanilyl-N_2-n-butylcarbamid (BZ 55) — in grösserem Ausmass zur Diabetesbehandlung angewandt. Der blutzuckersenkende Mechanismus dieses Stoffes ist noch nicht zu Genüge geklärt. Obgleich eine Wirkung auf den Blutzucker nur bei solchen Personen aufzutreten scheint, die in der Lage sind, selbst Insulin zu produzieren (3, 4), dürfte er sich grundlegend von dem des Insulins unterscheiden.

Nach Andersson (2) kommt es bei Diabetikern, die auf eine Behandlung mit BZ 55 ansprechen, zu einer deutlichen Senkung von Cholesterin und Gesamtlipoiden. Fuchs (7) hat jedoch festgestellt, dass die Serumcholesterinwerte in den ersten Wochen der Behandlung zwar vorübergehend abfallen, nach einem Vierteljahr aber wieder über den Ausgangswert hinaus ansteigen. Ähnliche Beobachtungen wurden auch von Munro und Murray (17) mitgeteilt. Einer von uns (G. H.) konnte nachweisen, dass BZ 55 auch bei Ratten ein Ansteigen des Serumcholesterins verursacht (8). Dieses steht vielleicht mit der thyreostatischen Wirkung von BZ 55 in Zusammenhang, die bei Menschen (6, 13, 16) und auch in Tierversuchen (1, 10, 14) festgestellt worden ist.

Wegen der möglichen Zusammenhänge zwischen Diabetes und Atherosklerose einerseits sowie BZ 55 und Fettstoffwechsel andererseits schien es von Interesse, die Wirkung von BZ 55 auf die experimentelle Atherosklerose am Tier zu untersuchen.

METHODEN

Für den Versuch wurden 42 sieben Wochen alte Leghorn-Hähnchen verwendet. Die Tiere erhielten Standardkückenfutter ad libitum, dem 2% Cholesterin und 5% Baumwollsamenöl bei-

gemischt waren. Die Hälfte der Tiere diente als Kontrollserie (Gruppe 1). Dem Futter der anderen Hälfte wurde 0,5% Alentin[1] = BZ 55 beigemischt (Gruppe 2). Als sich nach zwei Wochen herausstellte, dass diese Tiere ungefähr ⅓ weniger frassen als die Kontrollen, wurde der Cholesteringehalt des Futters der Gruppe 2 auf 3% erhöht, um eine mit der Kontrollgruppe annähernd übereinstimmende Cholesterinaufnahme zu sichern. Alle Hähnchen wurden zu Beginn und am Ende des Versuches gewogen. Nach 8 Wochen wurde sämtlichen Tieren Blut aus der Flügelvene zur Bestimmung des Blutzuckers, des Serumcholesterins und der Gesamtlipoide entnommen, wonach sie getötet wurden. Die inneren Organe wurden makroskopisch betrachtet. Herz und Aorta wurden herauspräpariert. Der thorakale Abschnitt der Aorta wurde in unverdünnter Sudan IV-Lösung gefärbt. Die atherosklerotischen Veränderungen wurden makroskopisch nach der von Katz (12) angegebenen Technik von 0 bis 4 beurteilt, wobei dem Beurteiler die Gruppenzugehörigkeit nicht bekannt war. Die Herzen wurden in Formalin (10%) fixiert und durch einen beide Kammern halbierenden Längsschnitt in zwei Hälften geteilt. Von jeder Hälfte wurden je zwei Gefrierschnitte senkrecht zur Längsachse des Herzens angefertigt. Dabei wurde ein Schnitt dicht unterhalb der Vorhöfe gelegt, der andere 2—3 mm apikalwärts. Die Schnitte wurden mit Sudan IV und Hämatoxylin gefärbt. Die in den von jedem Herzen hergestellten vier Präparaten mikroskopisch sichtbaren Koronargefässe wurden gezählt und der Prozentsatz der Gefässe mit deutlichen atherosklerotischen Veränderungen ermittelt. Auch hierbei war der Beurteiler über die Herkunft des Präparates nicht informiert.

Der Blutzucker wurde nach Somogyi (20) bestimmt, die Gesamtlipoide durch Färbung mit Sudanschwarz nach Swahn (24), indem jeweils drei Proben desselben Serums und gleich viele Serumproben von Kontroll- und Versuchstieren auf dem gleichen Papierstreifen gefärbt wurden. Das Serumcholesterin wurde nach der Methode von Schoenheimer-Sperry gemäss der Modifikation von Sperry und Webb (21) bestimmt. Mit Ausnahme der makro-

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[1] Alentin (WZ) ist identisch mit den in Deutschland im Handel befindlichen Präparaten Nadisan (WZ der Fa. C. F. Boehringer & Soehne, Mannheim-Waldhof) und Invenol (WZ der Farbwerke Hoechst, Frankfurt a.M.-Höchst). Alentin wird in Finnland von der Arzneimittelfabrik Lääketehdas Orion Oy mit der Lizenz von C. F. Boehringer & Soehne, Mannheim-Waldhof, hergestellt.

skopischen und mikroskopischen Beurteilung atherosklerotischer Gefässveränderungen wurden die erhaltenen Ergebnisse statistisch ausgewertet.

ERGEBNISSE

Während des Versuches starben ein Hähnchen der Kontrollgruppe und zwei Hähnchen der Gruppe 2, die BZ 55 erhielten. Der Nahrungsverbrauch der Gruppe 2 war während der ganzen Versuchszeit um ungefähr $\frac{1}{3}$ kleiner als der der Kontrollgruppe. Die

TABELLE 1

WIRKUNG DER ZUFÜTTERUNG VON BZ 55 ZU CHOLESTERINHALTIGEM FUTTER BEI HÄHNCHEN

	Kontroll-Gruppe (1) 20 Tiere	BZ 55-Gruppe (2) 19 Tiere
Mittelwerte der Tiergewichte zu Beginn des Versuches ± mittl. Fehler	648 ± 47,2 g	659 ± 42,0 g
Mittelwerte der Tiergewichte nach 8 Wochen ± mittl. Fehler	1.490 ± 66,0 g	1.150 + 48,3 g
Anzahl der Tiere mit Atherosklerose der Aorta	17 (85 %)	18 (95 %)
Anzahl der Tiere mit Veränderungen ≥ 1	15 (75 %)	17 (90 %)
Mittlerer Grad der Aortenatherosklerose für alle Tiere der Gruppe..	2,2	3,2
Mittlerer Grad der Aortensklerose bei Berücksichtigung lediglich der Tiere mit Veränderungen	2,6	3,4
Koronararterien mit deutlichen atheroskl. Veränderungen in % aller untersuchten Koronararterien	27 %	34 %
Mittelwerte des Blutzuckers nach 8 Wochen ± mittl. Fehler	170 ± 6,6 mg %	172 ± 10,6 mg %
Mittelwerte des Serumcholesterins nach 8 Wochen ± mittl. Fehler..	1.019 ± 161,3 mg %	1.327 ± 133,7 mg %
Mittelwerte der Gesamtlipoide des Serums nach 8 Wochen ± mittl. Fehler	1.962 ± 480,3 mg %	2.609 ± 538,2 mg %

Durchschnittsgewichte der Hähnchen zu Beginn und am Ende des Versuches sind in Tabelle 1 aufgeführt. Sowohl das mittlere Gewicht der Kontroll- als auch der Versuchstiere stieg während des Versuches deutlich an, bei den ersteren jedoch erheblich stärker. Die Differenz zwischen den mittleren Gewichten beider Gruppen am Ende des Versuches ist statistisch signifikant ($p < 0,001$).

Bei makroskopischer Betrachtung der abdominalen Organe fiel die häufigere blassgelbbraune Farbe der Leber bei den BZ 55-Tieren auf. Bei 4 Hähnchen dieser Serie fand sich im Herzbeutel reichlich klare Flüssigkeit, und das Perikardium erschien verdickt. Es handelte sich hierbei um die Tiere mit der geringsten Gewichtszunahme.

Die makroskopische Beurteilung der Aortensklerose ergab stärkere atherosklerotische Veränderungen der Gefässwand bei den Hähnchen, die zusätzlich BZ 55 bekommen hatten (Tab. 1). Alle Tiere mit Perikarderguss hatten atherosklerotische Veränderungen, die der Stufe 4 entsprachen.

Aus der mikroskopischen Untersuchung der Koronargefässe ging ungefähr gleich häufiges Auftreten von deutlichen atherosklerotischen Veränderungen in beiden Gruppen hervor. Bei Gruppe 1 waren 27% von rund 600 gezählten Koronargefässen atherosklerotisch verändert, bei der BZ 55-Gruppe 34%. Bei den 4 oben erwähnten Hähnchen mit Perikarderguss zeigte das histologische Bild Hyalinisation der Herzmuskelfasern, Degeneration der Muskelkerne und stellenweise Infiltrate von mononukleären und polymorphkernigen Zellen. Bei zweien dieser Tiere war das Epikardium verdickt und wies gleichartige Zellinfiltrate auf.

Blutzucker-, Cholesterin- und Gesamtlipoidwerte des Serums sind in Tabelle 1 aufgeführt. Zwischen den Blutzuckerwerten der Kontroll- und der BZ 55-Gruppe bestand kein Unterschied. Die Serumcholesterin- und Gesamtlipoidwerte der Gruppe 2 lagen etwas höher als die der Kontrollgruppe. Diese Unterschiede liessen sich jedoch nicht statistisch sichern ($p = 0,16$ bzw. $0,09$).

BESPRECHUNG

Die BZ 55-Dosis von 0,5% des Trockenfutters, welche in Abhängigkeit vom durchschnittlichen Futterverbrauch auf das mittlere Körpergewicht der Gruppe berechnet ungefähr 0,7 g/kg

während der ersten Wochen und etwa 0,9 g/kg am Ende des Versuches betrug, wurde auf Grund eines Vorversuches gewählt. Dieser hatte ergeben, dass die entsprechende Dosis bei 6 von 10 sieben Wochen alten Hähnchen eine deutliche Hypoglykämie hervorrief. Im vorliegenden Versuch zeigten die Hähnchen während der ersten Wochen entsprechendes äusseres Verhalten wie die hypoglykämischen Tiere im Vorversuch. Zum Ende des Versuches hin schwanden jedoch diese Symptome und der tägliche durchschnittliche Futterverbrauch stieg an. Obgleich die tägliche BZ 55-Aufnahme dadurch relativ grösser wurde, ergab die Untersuchung des Blutzuckers am Ende des Versuches bei BZ 55- und Kontrolltieren normale Werte. In Anbetracht der Besonderheit des Kohlenhydratstoffwechsels bei Hühnern, die sich u.a. darin zeigt, dass Entfernung des Pankreas keinen Diabetes verursacht (12), soll auf diese Fragen an dieser Stelle nicht näher eingegangen werden.

Weiterer Klärung bedarf auch die Frage, ob die Myo- und Perikarditis bei den 4 Tieren der Gruppe 2 auf die BZ 55-Medikation zurückzuführen ist. Myokarditische Veränderungen nach Sulfonamidverabreichung sind bei Tieren beobachtet worden (5).

Bei normalen Hähnchen tritt in diesem Alter noch keine spontane Atherosklerose im Bereich der thorakalen Aorta auf, wie wir in Zusammenhang mit einer anderen Untersuchung an 15 Wochen alten Hähnchen der gleichen Zucht feststellen konnten (Tabelle 2).

TABELLE 2

NORMALWERTE VON 10 HÄHNCHEN (ALTER 15 WOCHEN, STANDARDKÜCKENFUTTER)

Mittelwert der Tiergewichte ± mittl. Fehler ..	1.491 ± 88,1 g
Mittelwert des Serumcholesterins ± mittl. Fehler	74,8 ± 4,6 mg %
Mittelwert der Gesamtlipoide ± mittl. Fehler ..	249 ± 32,5 mg %
Anzahl der Tiere mit Atherosklerose der Aorta	0

Bei Ernährung mit cholesterinhaltigem Futter geht das Auftreten von atherosklerotischen Veränderungen der Aorta dem Ansteigen der Serumcholesterinwerte parallel (12). Es liegt deshalb nahe, die in unseren Versuchen bei Zugabe von BZ 55 beobachtete Verstärkung der durch Cholesterinfütterung bedingten Aortenatherosklerose mit den gleichzeitig erhaltenen höheren Serumcholesterin- und Gesamtlipoidwerten in Zusammenhang zu bringen. Dabei

bleibt das Fehlen entsprechender Unterschiede im Befall der Koronargefässe bemerkenswert. Auch die bei Tieren der BZ 55-Gruppe häufigere Blassgelbbraunfärbung der Leber mag mit den Differenzen der Serumlipoidwerte zusammenhängen. Da sich jedoch die Unterschiede in den Serumcholesterin- und Gesamtlipoidwerten zwischen BZ 55- und Kontrollgruppe statistisch nicht sichern lassen, sind solche Rückschlüsse nur mit grosser Zurückhaltung möglich. Dazu kommt noch, dass die Hähnchen der beiden Gruppen während des Versuches pro Tier zwar im Durchschnitt die gleiche Gesamtmenge Cholesterin aufnahmen (126 g/Tier in Gruppe 1 und 125 g/Tier in Gruppe 2); pro kg mittleres Körpergewicht am Ende des Versuches berechnet, hatten die BZ-Hähnchen jedoch durchschnittlich 111 g/kg und die Kontrolltiere 86 g/kg gefressen. Bei Berücksichtigung der Ergebnisse von Katz (12) über die Wirkung von Versuchsdiät mit verschiedenem Cholesteringehalt, scheint es allerdings unwahrscheinlich, dass die Differenz in der Cholesterinaufnahme die von uns beobachteten Unterschiede verursacht haben könnte. Für die Möglichkeit, dass hierbei doch eine Wirkung von BZ 55 deutlich wird, spricht die Beobachtung, dass BZ 55 Serumcholesterin und Gesamtlipoide unter entsprechenden Bedingungen auch bei der Ratte erhöht (8). Auch bei Diabetikern ist festgestellt worden, dass die Cholesterinwerte des Serums bei langdauernder Medikation von BZ 55 nach anfänglichem Abfall wieder auf den Ausgangswert und darüber hinaus ansteigen (7, 17). Ausserdem liegen eigene Beobachtungen späteren Datums vor, wonach Verabreichung von BZ 55 sowohl bei gewöhnlichem Futter den Serumcholesterinspiegel von Hähnchen hebt, als auch nach Absetzen von Cholesterinfütterung das Absinken des Serumcholesterins auf normale Werte verzögert.

Trifft es zu, dass BZ 55 in der vorliegenden Untersuchung eine Erhöhung des Serumcholesterins und dadurch ein verstärktes Auftreten von Aortenatherosklerose verursacht hat, so könnte dieses vielleicht mit der thyreostatischen Wirkung von BZ 55 zusammenhängen, die bei Versuchstieren (1, 10, 14) und auch beim Menschen festgestellt worden ist (6, 13, 16). Hierfür könnte auch der geringere Gewichtsanstieg bei den Tieren der Gruppe 2 in der vorliegenden Untersuchung sprechen.

Wie weit andere, mit der blutzuckersenkenden Wirkung von BZ 55 zusammenhängende Mechanismen als Ursache der hier ange-

führten Beobachtungen in Betracht zu ziehen sind, ist zum gegenwärtigen Zeitpunkt nicht zu entscheiden. Auffallend ist eine gewisse Parallele zum Effekt von Insulin. Zufuhr von Insulin und gleichzeitige Verfütterung von Cholesterin bewirkt bei nichtdiabetischen Hähnchen zwar keine zusätzliche Erhöhung des Serumcholesteringehaltes, jedoch ein deutliches Mehrauftreten von atherosklerotischen Veränderungen an Aorta und Koronarien (23). Der Unterschied im Verhalten von Serumlipoiden und Koronarsklerose macht es jedoch zweifelhaft, ob die atherogenen Wirkungen von BZ 55 und Insulin auf dem gleichen Mechanismus beruhen.

Die erhaltenen Beobachtungen geben einen Hinweis, dass die Erhöhung der Serumcholesterin- und Gesamtlipoidwerte und das Auftreten atherosklerotischer Veränderungen der thorakalen Aorta infolge Cholesterinzufütterung bei Hähnchen durch Zugabe von BZ 55 verstärkt werden könnte. Eine endgültige Stellungnahme wird erst auf Grund weiterer Untersuchungen möglich sein.

ZUSAMMENFASSUNG

Bei 7 Wochen alten Hähnchen verursachte die Zufütterung von BZ 55 über 8 Wochen in relativ sehr hohen Dosen zu cholesterinhaltigem Futter verminderten Gewichtsanstieg, mässige Steigerung der Serumcholesterin- und Gesamtlipoidwerte und stärkere atherosklerotische Veränderungen der Aorta, jedoch keine stärkere Koronarsklerose im Vergleich zur Kontrollserie mit Verfütterung von Cholesterin allein. Mit Ausnahme der Gewichtsdifferenz waren die Befunde jedoch statistisch nicht zu sichern.

Die Vermutung einer atherogenen Wirkung von BZ 55 an Hähnchen — wie sie von anderen Autoren auch für zugeführtes Insulin unter ähnlichen Bedingungen nachgewiesen wurde — bedarf weiterer experimenteller Sicherung.

LITERATUR

1. ACHELIS, J. D. und HARDEBECK, K.: Dtsch. Med. Wschr. 1955:*80*:1452.
2. ANDERSSON, B.: Sv. Läkartidn. 1956:*53*:1213.
3. BERTRAM, F. BENDFELDT, E. und OTTO, H.: Dtsch. Med. Wschr. 1955:*80*:1455.
4. BEST, CH. H.: Canad. Med. Ass. J. 1956:*74*:957.
5. BOYD, W.: Textbook of Pathology, Henry Kimpton, London, 1947.

6. DUNCAN, L. J. P., JOYCE, D. P. und DUNLOP, D. M.: Brit. Med. J. 1956:*II*:433.
7. FUCHS, J.: Münch. med. Wschr. 1956:*98*:1103.
8. HÄRTEL, G. und ANTILA, V.: Arzneim.-Forsch. 1956:*6*:701.
9. HEINSEN, H. A., in Klinik der Gegenwart, Band 2, Urban & Schwarzenberg, Berlin-München, 1956.
10. v. HOLT, C., KRACHT, J., KRÖNER, B. und v. HOLT, L.: Schweiz. med. Wschr. 1956:*86*:1123.
11. JOSLIN, E. P.: J.A.M.A. 1949:*139*:1.
12. KATZ, L. N. und STAMLER, J.: Experimental Atherosclerosis, Am. Lect. Ser., Thomas Publ., Springfield, 1953.
13. KINSELL, L. W., BROWN, F. R., FRISKEY, R. W. und MICHAELIS, G. D.: Science 1956:*123*:585.
14. KUUSISTO, A. N. und ANTILA, V.: Acta Endocrinologica 1956:*23*:433.
15. LIEBOW, I. M. und HELLERSTEIN, H. K.: Am. J. Med. 1949:*7*:660.
16. McGAVACK, T. H., SEEGERS, W., HAAR, H. und ERK, V.: Metabolism 1956:*5*:919.
17. MUNRO, I. B. und MURRAY, D.: Lancet 1956:*271*:1083.
18. ROOT, H. F. und WILSON, J. L.: Circulation 1950:*2*:474.
19. SCHETTLER, G., in Klinik der Gegenwart, Band 2, Urban & Schwarzenberg, Berlin-München, 1956.
20. SOMOGYI, M.: J. Biol. Chem. 1952:*195*:19.
21. SPERRY, W. M. und WEBB, M.: J. Biol. Chem. 1950:*187*:97.
22. STAMLER, J., PICK, R. und KATZ, L. N.: Circulation 1955:*12*:487.
23. STAMLER, J., PICK, R. und KATZ, L. N.: Circulation 1956:*14*:492.
24. SWAHN, B.: Scand. J. Clin. Lab. Invest. 1953: 5, Suppl. 9.

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EFFECTS OF RADIATION ON ONE OF TWO YOSHIDA SARCOMAS TRANSPLANTED INTO THE SAME ANIMAL[1]

by

ANTERO VOUTILAINEN and VÄINÖ K. HOPSU

A previous study showed that, when one of two ITB tumours transplanted into a rat was treated with roentgen rays, the irradiated tumour disappeared, but the growth of the other, non-irradiated tumour was also much slower compared with control rats, the difference being statistically significant (6). It was assumed that some growth-inhibiting factor is liberated from the irradiated tumour and that other parts of the body are then affected humorally. According to Gaspari (1), such a necrohormone in high concentration is a cell toxin having a cytolytic action on the cell.

The purpose of this work was to study the question further using another kind of tumour and a larger number of rats, the transfer being performed into different tissue. The malignant Yoshida sarcoma characterized by rapid intramuscular growth chosen for the study. It was decided to inject the animals at two sites and study the remote action of the tumour-inhibiting factors, released by topical irradiation, on the non-irradiated one of these homologous tumours.

MATERIAL AND METHODS

Yoshida sarcoma (7, 8) which grows well for example in rat muscle and is easily transplantable from one experimental animal into another, was used for the study. Sarcoma obtained from a rat's hind leg muscle was pulped in a mortar and suspended in saline. Using a fine needle, about $\frac{1}{2}$ cc. of the suspension was injected into each experimental animal. With this dose tumours were always transplanted.

The experimental animals were 3-month-old male withe rats and totalled 40. The suspension was injected into the crural muscles of the right fore leg and left hind leg. Obvious and palpable tumour developed at these sites in 6 days. Biopsy specimens were now taken from the hind leg tumours of 20 rats. Two days later irradiation was started: a time dose of 500 r to a depth of $\frac{1}{2}$ cm. was administered for 10 successive days using the Stabilivolt-Siemens equipment of the Central Institute of Radiotherapy (160 kW, 10 mA, a 5 Al filter, distance 20 cm., radiation field 3×6 cm.). The $\frac{1}{2}$ cm. depth dose was 200 r/min. and the H.V.L. 0.35 mm. Cu. During treatment the rats were under ether anesthesia with their limbs attached to a frame to prevent them from moving on waking up. Apart from the fore leg tumour, the rats were shielded during irradiation by 3 mm. thick lead sheet, which according to measurements sufficed to protect the other parts of the trunk and the left hind leg against irradiation. The remaining 20 rats were kept as controls.

Following each irradiation, the tumours in the fore and hind legs of both the treated and the control rats were measured in three directions always by the same method, and the rats were weighed (the product of the dimensions is here termed volume). No rats was deliberately sacrificed. Specimens biopsy were taken from all tumours after death.

EXPERIMENTS AND CONCLUSIONS

The mean volumes of the tumours of both the irradiated and the non-irradiated rats were calculated for the different days. The growth variations of the various tumours seen in the figure 1 were thus obtained.
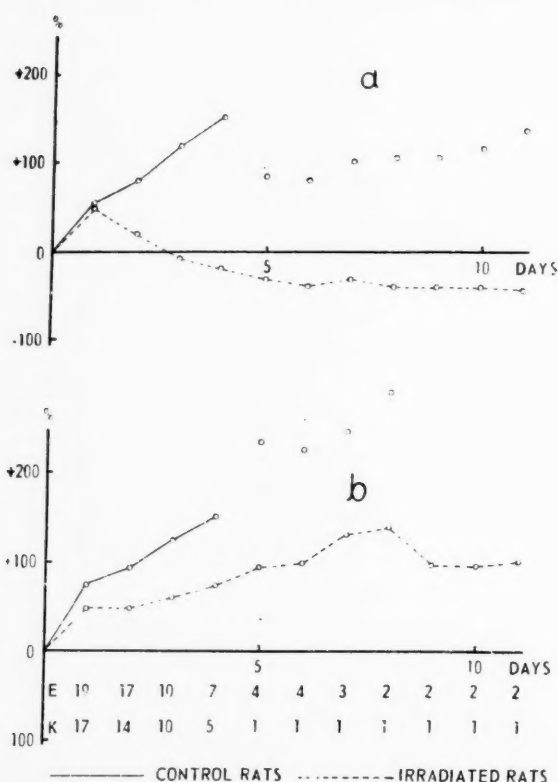
Fig. 1. — Diagrams showing the per centage growth variations of tumours compared to tumour volumes of the first day of irradiation. a = fore leg tumours, b = hind leg tumours. E = Number of surviving irradiated rats. K = Number of surviving controls. o = the per centage tumour volumes of the still surviving single control rat.

Diagrams show the growth variations of Yoshida sarcomal calculated as average volumes during and after irradiation untis death. The number of surviving rats on each day is indicated below the curves.

It is seen that the volume of the irradiated tumours decreased daily as would be expected. The tumour volumes were of the order of 10 cc. at the commencement of treatment. The growth variations of all non-irradiated tumours were similar and a tendency to growth was present. It is found, however, that the vigorous growth from the first day on occurred in the hind leg tumours of the non-irradiated controls, and that, in comparison, growth was distinctly

slower in the non-irradiated hind leg tumours of the irradiated rats. At 10 days the volume curve for the non-irradiated tumour of the irradiated rats showed a considerable decrease. However, this seems to be due largely to concurrent death of animals, and thus does not give a entirely correct picture of the growth of these tumours at this time. In this group the tumour volume seems to increase continuously until the end of the experiments.

As already stated, each group included 20 rats at the beginning of the experiment, i.e. 20 rats to be treated and 20 controls. On the fourth day 10 rats survived in each group, but on the sixth day 4 rats survived in the irradiated group and only one in the non-irradiated; the figures at the end of the experiment were 2 and 1 respectively. In both groups 1 rat survived for 25 days. Of the irradiated rats, 3 died during anesthesia, on the second, fourth, and seventh days. The others were in poor general condition declined and died of inanition.

Biopsies were taken from the non-irradiated hind leg tumours of the irradiated rats before the beginning of treatment to ensure the presence of a malignant tumour. The histologic picture showed intense infiltrating growth between the muscle fibres. In places there were true tumour cells either densely arranged or far apart. Necrotic islets were seen here and there. Inflammatory reactions were not usually observed. The mitoses were counted and numbered 20—30 per 2,000 cells.

Post-mortem biopsy specimens were removed from the fore and hind leg tumours of both the irradiated and the control rats. The irradiated tumours were found on histological examination to be completely necrotic and disappeared. The non-irradiated tumours of the same group showed necrosis of extremely high degree in 9 cases, the tissue being entirely destroyed in 2 specimens. In 2 cases living tumour cells were present in large numbers but there was little necrosis. In the other cases tumour tissue and necrosis were about equal amount. Attention was paid to mitoses but they were not counted, as the results would not have been comparable owing to the rats having died at different times. Mitoses were found in 13 cases. One specimen showed, in addition to abundant necrosis, distinct cytolysis of cells (Table 1).

Partially necrotic tumour tissue was present in the histological picture of the control group too, and both in the fore and the hind

TABLE 1

HISTOPATHOLOGICAL APPEARANCE OF YOSHIDA SARCOMA AT DEATH OF RATS.
FIGURES = NUMBER OF EXPERIMENTAL ANIMALS

		No	Slight	Moderate	Intense	Highly Intense
Non irradiated hind leg tumours of irradiated rats	Necrosis	0	5	4	7	2
	Mitotic frequens	8	7	3	0	0
Control	Fore leg tumours	Necrosis	0	4	8	6	1
		Mitotic frequens	5	12	2	0	0
Rats	Hind leg tumours	Necrosis	0	6	6	6	1
		Mitotic frequens	6	13	0	0	0

leg. One specimen of fore leg and one of hind leg tumour showed total necrosis. The greater part of the tissue was necrotic in 6 fore leg and 6 hind leg tumours. There were viable tumour cells in abundance but little necrosis in 3 fore leg and 5 hind leg tumours; in the rest an equal amount of tumour tissue and of necrosis occurred. Ten hind tumours in this group showed necrosis.

As appears from diagramms a and b, the most vigorous growth tendency was observed in the hind leg tumour of the control group during the period when the animals of the other group received irradiation. Growth of the non-irradiated tumour in the group of irradiated rats was found to have decreased as early as the second day of treatment. Thus similar tumour regression was found in these non-irradiated tumours as in a previous study (6), in which one of two homologous tumours was irradiated. Injection of tissue suspensions obtained from irradiated animals also resulted in regression of malignant tumours (5), and on this basis it was suggested that a growth-inhibiting factor may be released in the form of a necrohormone, which — in high concentration — has a cytolytic effect on the tumour cell (1). In the present experiments there was abudant cytolysis at least in one case in the non-irradiated tumour of an irradiated rat, and in general the tendency to necrosis was greater in the non-irradiated tumours of the irradiated group than in the corresponding control tumours.

Roffo (1938) produced tumour regression and disappearence

injecting extracts of hydrolyzed striated muscle into rats bearing fibrosarcoma (4). He did not observe the same phenomenon when using extracts of striated muscle. Helff (1948) injected extracts made from degenerating tail skin of involuting *Rana catesbeiana* larvae directly into spontaneous mammary tumours of mice. He noted considerable retrogression (2). Extracts made from histolyzing gills of artifically metamorphosed *Rana clamitans* larvae also producted pronounced regression of mammary tumours (3). These experiments show that necrotic or necrotizing tissue has a tumour inhibiting effect.

In the present work none of the experimental animals were cured, and 13 days after the beginning of the experiment only one rat survived. This last survivor also exhibited fairly rapid tumour development. Histological examination showed highly invasive intramuscular growth.

The fifth and sixth days seem to be critical: at this time many deaths occurred in the control group. In the irradiated group, however, the rats on an average died later than in the control group. This is probably accounted for partly by irradiation: it caused disappearance of one tumour and, probably by releasing a tumour inhibiting factor, regression of the other this latter fact — naturally — also lengthens the survival time. As pointed out earlier (5) is the immunological background in these experiments of great importance. In this study, however, no serological examinatioes were made. Such experiments are in progress in our laboratory now.

The invasive intramuscular growth of the Yoshida sarcoma renders exact evaluation of tumour size difficult. Owing to this fact, and also to the fairly low mitotic activity of this tumour and to the early death of the experimental animals, Yoshida sarcoma does not seem to be a very suitable tumour for experimental studies of this kind.

SUMMARY

1. Forty 3-month-old male white rats, simultaneously inoculated with Yoshida sarcoma, were used for the study; the site of injection was the crural region of the right fore leg and the left hind leg. One week after injection, irradiation was commenced:

the tumour-bearing area of the fore leg of 20 rats was irradiated with daily doses of 500 r, the total dose being 5000 r. The remaining 20 rats served as controls.

2. Complete abolition of the irradiated tumour took place during treatment. The non-irradiated tumours of the irradiated rats grew more slowly than the corresponding tumours in the control group. After death the histological picture of the former group showed more necrosis than was seen in the latter group. The average survival time of the irradiated rats was longer than that of the controls.

3. The slower growth of the non-irradiated tumours of the irradiated rats is attributed to the possible release of tumour inhibiting agents from the necrotic tumours.

REFERENCES

1. Gaspari, W.: Arch. klin. Chir. 1927:*146*:711.
2. Helff, O. M.: Proc. Soc. Exp. Biol. and Med. 1928:*69*:336.
3. Helff, O. M.: Proc. Soc. Exp. Biol. and Med. 1955:*88*:135.
4. Roffo, A. H.: Biol. Inst. Med. Exp. (Buenos Aires) 1938:*14*:257.
5. Teir, H., and Voutilainen, A.: Acta Pathol. et Microbiol. Scand. 1957:*40*:273.
6. Voutilainen, A.: Acta Radiol. 1957:*47*:149.
7. Yoshida, T.: Gann 1949:*40*:1.
8. Yoshida, T.: J. Nat. Cancer Inst. 1952:*12*:947.

TRANSPOSITION OF THE SPLEEN INTO THE THORACIC CAVITY

EXPERIMENTAL OBSERVATIONS: PRELIMINARY REPORT

MARTTI TURUNEN, HEINO LAITINEN, MIKKO PASILA
and L. F. STJERNVALL

There are numerous experimental studies and clinical investigations on the reduction of pressure in portal hypertension. It is a common feature in all methods that attempts are made to direct the blood flow from the portal area, either by a shunt or via a developing collateral circulation past the hepatic circulation. In a paper published in 1955 Nylander and Turunen described the transplantation of the spleen into the thoracic cavity in three patients with bleeding oesophageal varius, which method was based on incidental findings in connection with war injuries. The bleeding from the oesophageal varices was arrested. The spleen had obviously formed adherences to the wall of the thoracic cavity and created a collateral circulation past the liver into the vena cava. However, there was no reliable clinical evidence nor any experimental study in the matter. The writers have carried out similar investigations on dogs, but a detailed analysis has not yet been completed and will be published at a later date. We did not attempt by our method to produce hypertension over the whole portal area in the dog, but only in the splenic circulation. Our main problem was to what extent the surplus blood of the enlarged and congested spleen sought to flow elsewhere, also how many anastomoses were created and in which direction. To achieve this purpose

the splenic veins were ligated either completely or partly, and only the lienal artery was left intact, and the spleen was grafted into the thoracic cavity.

MATERIAL AND METHOD

Thoracolaparotomy was carried out on nine dogs. The splenic veins were partly ligated completely in some of the dogs, and in others one functioning vein was left open to convey blood to the portal area. To promote the formation of adherences asbestos powder was placed around the spleen in thoracic cavity of 8 dogs, and finely-ground bone marrow in one, together with fluid squeezed out of bone-marrow, which was supposed to favour formation of granular tissue. The immediate mortality was four dogs. Five dogs stayed alive and remained well under the usual treatment and diet for a period of 2—4½ months. The dogs were then operated again. The thorax was opened and 20 ccm contrast medium was injected into the spleen (Urografin 76% Schering A. G. Berlin) and an X-ray photograph was taken. The dogs were then autopsied and histological samples were taken.

RESULTS

Macroscopically the spleen was atrophied in all the dogs. It was about one third of the normal size and colourless. The cross section looked fibrotic and brownish. A common feature was more-over that it had formed in all cases close adhesions to the diaphragm and to the adjacent surface of the lung, but, with the exception of one dog, there were no noteworthy adhesions to the thoracic wall. We could roentgenologically demonstrate functioning blood vessels going in the direction of the thorax cavity and pericardium, and in the neck into the subclavian vein (fig. 1, 2). In those dogs in whom one functioning vein had been left in the direction of the portal area, it was dilated and functioned in the main part as a reducer of splenic hypertension. But in those dogs in which no communication with the former circulation had been left, the blood made for itself a passage along the created veins into the thoracic wall and particularly along a new vein, which passed from the splenic hilus following the phrenic nerve into the neck and the left subclavian vein (fig. 2, 3). An anastomosis had also formed downwards, into the area of the mesenteric vein (fig. 2).
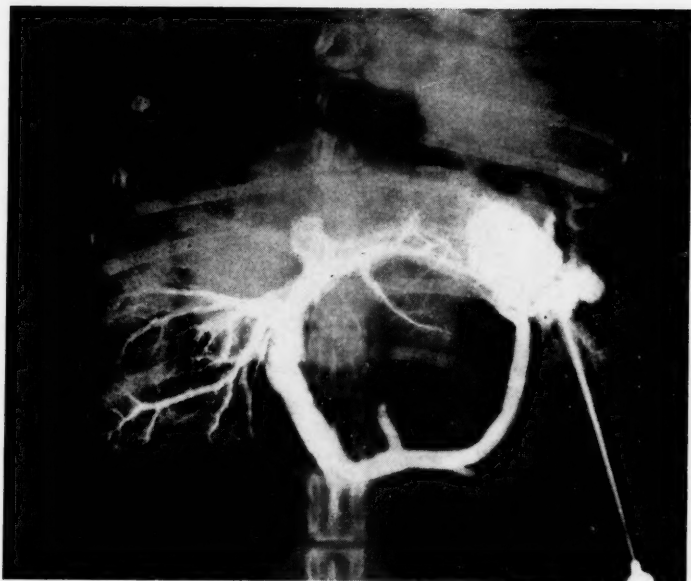
Fig. 1. — Splenoportography. The spleen is seen in the thoracic cavity. Some of the veins of portal circulation are ligated. The splenic and portal veins are normal. The varicose veins are passing from the spleen into the pleura.
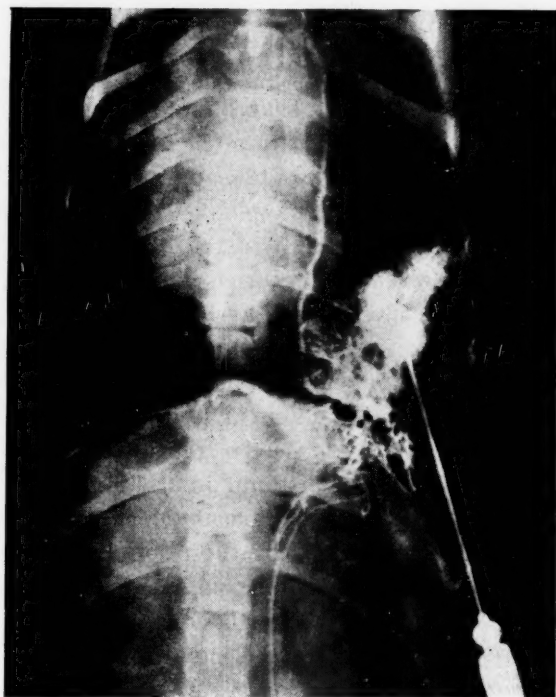
Fig. 2. — Splenoportography. The spleen is seen in the thoracic cavity, with dilatation of its circulation and atrophy of its pulp. The splenic vein is ligated, the portal vein is not filled. A large vein passes from the spleen up into the subclavian vein, another doun into the mesenteric circulation.
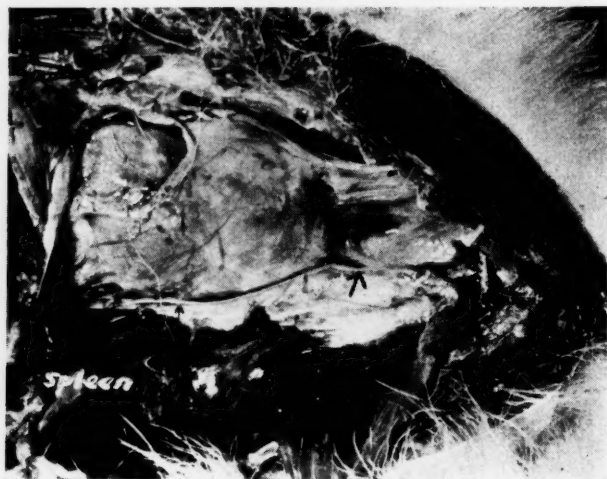
Fig. 3. — The same case as in fig. 2. A large vein is passing beneath the phrenic nerve up into the subclavian vein from the spleen in the thoracic cavity.

DISCUSSION

An interesting feature of the results is in the first place the formation of new blood vessels. A collateral circulation was created communicating with the pulmonary tissue and the wall of the thoracic cavity, which seems natural in the light of Nylander's clinical results. Unexpected was the appearance of anastomoses, beginning with the hilus of the spleen imbedded into the diaphragm, and along the phrenic nerve, up to the left subclavian vein. In other words, the formation occurred between two completely separate venous systems, with the anastomosis directed along the phrenic nerve. Secondly, interest is focused on the behaviour of the spleen after its transference from its normal site. The roentgenogram shows that its internal circulation dilatated (fig. 2) and macroscopically it could no longer be recognised as splenic tissue. It is possible that it had changed into an arteriovenous shunt with the aim of diverting the excessive blood, which was aided by conditions of thoracic pressure and by the respiratory movements of the embedded lung. In some of the experimental animals a portion of the spleen had remained in the abdominal cavity. The portion situated in the thoracic cavity was then atrophied, colourless, but

the portion in the abdominal cavity was macroscopically functioning splenic tissue of normal appearance. In those animals in which a functioning vein had been left, the surplus blood of the spleen was drained off that way, and the formation of extra veins was considerably poorer (fig. 1). Asbestos proved to be a substance very strongly promoting adhesions, whereas bone marrow was considerably less affective in this respect. Great loss of animals occurred in those experiments, in which all splenic veins were completely ligated.

SUMMARY

In nine dogs the spleen was grafted into the thoracic cavity and the veins from the splenic hilus to the portal area were ligated either completely or partially. An abundant formation of collateral circulation was observed in the lung, the wall of the thoracic cavity and the neck. The veins were visualised roentgenologically and anatomically. Histological examination and a closer analysis of the results will be published in connection with subsequent experiments.

REFERENCES

NYLANDER, P. E. A., and TURUNEN, M.: Ann. Surg. 1955:*142*:954.

REVASCULARISATION OF THE HEART MUSCLE FOLLOWING THE TRANSPOSITION OF THE SPLEEN AND OMENTUM INTO THE THORACIC CAVITY

EXPERIMENTAL STUDY ON DOGS: PRELIMINARY REPORT

by

MARTTI TURUNEN, HEINO LAITINEN, MIKKO PASILA
and L. F. STJERNVALL

An increase of the blood supply to the heart muscle damaged by coronary diseases has been the object of research over a number of years. The following possibilities exist: either anastomosis is established direct between the heart muscle and some source outside of the heart, or new blood is introduced by means of a shunt into the coronary arteries themselves (1, 3). Numerous experiments have been made, but they are rarely applicable to clinical use. O'Shaughnessy (2) in 1936 grafted omentum into the pericardial cavity, at first in experimental animals and later in 57 human patients, with success. In the course of our animal experiments of transplanting the spleen into the thoracic cavity, the observed changes in the spleen and the formation of a strong collateral circulation in the thoracic region, gave rise to the idea of using the spleen as an arterio-venous shunt. Our tests showed in fact that the spleen became atrophied if it was grafted into the thoracic cavity, the functioning splenic tissue disappeared and it was obviously transformed into an organ for the passage of the blood, which was aided by respiratory movements of the lung. We conceived the idea that the veins returning from the spleen to the portal region, grafted

into the denuded heart and followed by the omentum, could be made
to adhere to the heart muscle and to establish anastomosis with
the coronary system. With this aim in view, we carried out a series
of experiments on dogs.

MATERIAL AND METHOD

Thoracic laparotomy was performed on nine dogs. The omen-
tum going from the greater curvature of the stomach into the
splenic hilus was separated along the whole greater ventricular
curvature. The artery and the vena lienalis were left untouched
and both spleen and omentum were brought through the dia-
phragm into the thoracic cavity. The pericardium was opened at
the site of the phrenic nerve and the omentum was sutured to the
pericardium in the region of the left cavity. In eight dogs powdered
asbestos was placed between the pericardial omentum and the
cardiac muscle, and in one dog bone marrow, in order to produce
adhesions. Immediate mortality at operation was five dogs, but the
rest remained alive and kept well under the usual treatment. After
the lapse of 2—4 months the animals were again operated on.
The spleen was exposed through thoracoabdominal incision, contrast
medium was injected into it and roentgenograms were made of the
dogs. They were then killed and autopsied.

RESULTS

It could be seen on the roentgenograms that the blood vessels
had remained open and the contrast was finding its way into the
thoracic cavity and the omental blood vessels (Fig. 1); except of
course the main portion of the contrast which passes along the vena
lienalis into the portal region. The spleen was atrophied and con-
stricted to one third of its original size. The cross section was colour-
less and did not resemble splenic tissue. The omentum was firmly
adherent to the heart muscle, and the pericardium likewise, nor
could it be detached along its anatomic boundaries. Macroscopically
the omentum was greatly abundant in blood vessels, as also shown
by the roentgenological examination. Adhesions of the omentum
to pulmonary tissue were found in one dog. Histological specimens
were taken, and a more detailed analysis of the results will be
published in connection with further experiments.
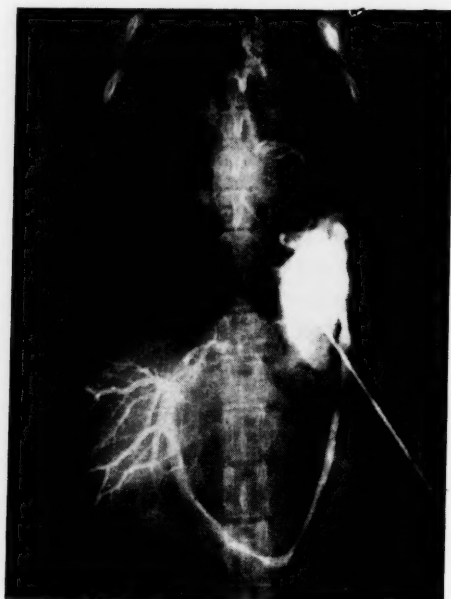
Fig. 1. — Splenoportography. The spleen is seen in the thoracic cavity, and an omental graft is sutured to the opened pericardium. The omental veins in the graft are filled with contrast medium. Collateration between the lienal and the coronary circulation is also in evidence.

DISCUSSION

Numerous experiments are carried out for a revascularisation of the heart. It is of interest in our series that we were successful in producing immediate adhesions between functioning blood vessels and the heart muscle. On roentgenogram (Fig. 1) we can see two blood vessels loop direct on the heart. These anastomoses which, with a high degree of probability, have established communication with the heart muscle, will be dealt with later, as well as the possible anatomic and functional changes brought about in the heart muscle by the omentum. Nevertheless, the pumping mechanism of the thorax cavity are able to keep the omental vessels open, and it is obvious that the blood must continue with its circulation, since adherences could not be macroscopically established elsewhere. Otherwise it is evident that thrombosis of the blood vessels would be bound to occur. We noted in our series

that powdered asbestos is a much more efficient agent for pro-
ducing adhesions than bone marrow. A great loss of animals
occurred in those experiments, in which all splenic veins were
ligated.

SUMMARY

Omentum was transplanted together with the spleen into the
thoracic cavity. Omentum was detached from the great curvature
of the ventricle and sutured to the edges of the opened pericardium.
The formation of adhesions was promoted by asbestos. It was then
noted that the blood vessels in the vicinity of the heart were open.
A copious formation of blood vessels in the omentum surrounding
the heart and in the pericardium was demonstrated roentgenologic-
ally and established macroscopically. The results of histological
studies will be reported in a comming paper.

REFERENCES

1. BECK, C. S.: IV Internationaler Kongress für Erkrankungen der Thorax-
      organe 1956.
2. O'SHAUGHNESSY, L.: Brit. J. Surg. 1936:23:665.
3. BAILEY, C. P.: Surgery of the Heart. Lea & Febiger, Philadelphia 1955.

FROM THE DEPARTMENT OF PATHOLOGY, SECTION II, AND THE DEPARTMENT OF OTOLARYNGOLOGY, UNIVERSITY OF HELSINKI

NUCLEOLAR VARIABILITY IN BENIGN AND MALIGNANT PAPILLOMAS OF THE LARYNX IN ADULTS

by

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(Received for publication March 12, 1957)

Over a hundred years ago the development of the microscope had reached a stage permitting measurement of cells, nuclei and nucleoli. Ever since then the question of the value of such measurements has been discussed, and there has been much controversy over the subject. In his monograph, »The Size and Growth of Tissue Cells», Hoffmann (7) describes in detail the technical and biologic causes of the sharp divergence of opinion, dealing with the difficulties associated with cytologic measurements.

MacCarty represents the most extreme views among the investigators who have studied this question during the last few decades. In a comprehensive report (11) of his numerous studies on fresh tissue in frozen section, he states that the nucleolus is much larger in proportion to the size of the nucleus in all malignant cells, regardless of the type or origin of the neoplasm. Guttman and Halpern (6) from their studies reached an entirely opposite opinion. They observed no essential differences between the nuclear-nucleolar volume ratios of normal tissue, benign and malignant tumours. Moreover they concluded that the volume of nucleoli of normal tissue is significantly smaller than the volume of nucleoli of tumours, finding, however, no difference between the nucleolar volume of benign and malignant tumours.

Cowdry (5) demonstrated that the nucleolus is larger in some malignant cells than in some non-malignant ones, yet he considered it unjustifiable and dangerous to attempt to reach a diagnosis of malignancy on the size of the nucleoli alone.

Long and Taylor (10) studied the nuleolar variability of gynecological tumours of different histological grade of malignancy; they found that for example the following nucleoler variations appeared with increased grade of malignancy: Multiple nucleoli; larger size of single nucleoli; variations in size of multiple nucleoli.

At the Department of Pathology of the University of Helsinki, measurements of nuclei and nucleoli have been made in several clinical and histological studies of tumours in various organs (3, 4, 8, 9, 12, 13, 14). The general conclusion has been that large nuclei and nucleoli occur in malignant tumours. Two of the above studies were concerned with papillomas. Stenius (14) studied benign and malignant papillomas of the bladder and noted that the nucleoli, to a greater extent than the nuclei, showed variations with increasing grade of malignancy. In the case of papillomas of the nose Saxén (12) found great nuclear and nucleolar size variation only in some cases.

A perusal of the literature has not revealed any data based on cytological measurements in cases of papillomatous tumour of the larynx.

MATERIAL AND METHODS

The cases on which the present investigation is based were admitted to the Otolaryngological University Clinic of Helsinki in 1949 to 1951 and represent a part of the series previously analyzed clinically and histologically (2). The present material consists of 3 beingn and 3 malignant laryngeal papillomas. In the benign group the ages of the patients at onset of symptoms were 35, 49 and 59 years; in the malignant group 54, 59 and 66 years. Of the benign papillomas, two were of the single type and one was multiple. The malignant papillomas were all single.

Not only the histological finding but also the clinical course was taken into account when classifying the cases as benign or malignant. The clinical evidence of benignancy required was freedom from symptoms for at least three years after endoscopic excision without any other treatment.

The three preparations of malignant tumour tissue included areas regarded as benign on the basis of the ordinary histological examination. Nuclear and nucleolar measurements were made on these areas separately.

The results to be presented here are based on material obtained at the first biopsy examination of each patient.

All specimens were fixed in a solution having the following composition: Saturated mercuric çhlorid 40.0; Formalin 10.0; Glacial acetic acid 2.0. The duration of fixation is not recorded in the laboratory journals. For the present investigation the preparations were sectioned at 5μ. The staining method adopted was hemalum-eosin.

With the aid of mirrors attached to the microscope the image of the cells was projected to a magnification of 2,540 times. The outline of the nuclei and nucleoli were drown on paper, and measurements done from these drawings. The longest and shortest diameter were measured in each case. If the nucleus or nucleolus was of irregular shape, the outline was adjusted to the shape of a circle or an ellipse giving approximately the same area as the original figure, and measurement was then done. The measurements stated in the tables are averages of the two diameters mentioned, and thus represent the mean areal diameter in μ.

Five hundred nuclei, and the nucleoli visible in them, were measured from the specimens of the benign cases. An equal number of nuclei and nucleoli were measured from both the malignant and the »benign» areas of the malignant preparations.

RESULTS

The number of nucleoli per nucleus was as follows:

TABLE 1

BENIGN AND MALIGNANT LARYNGEAL PAPILLOMAS. AVERAGE NUMBER OF VISIBLE NUCLEOLI PER NUCLEUS

			Malignant area	»Benign» area
Benign:	Case 1	2.29		
»	2	1.46		
»	3	1.48		
Malignant:	Case 4		1.66	2.10
»	5		1.60	2.15
»	6		2.44	2.08

The results of nucleolar measurements are presented in figures 1 and 2. These also show the mean maximal diameter in each case, *i.e.* the average of the ten largest measurements.
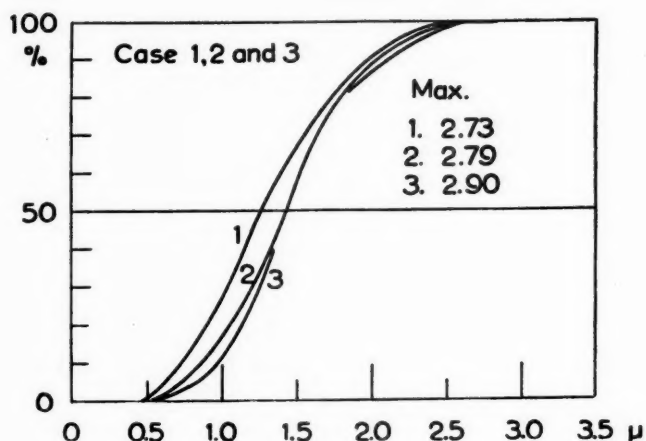
Fig. 1. — Benign laryngeal papillomas. Cumulative distribution curves of mean areal diameters of nucleoli. Mean maximal areal diameters of nucleoli.

The nuclear-nucleolar mean areal diameter ratio has been calculated by utilization of the second and the third quartiles from figures 1 and 2 and the corresponding nuclear measurements.

TABLE 2

BENIGN AND MALIGNANT LARYNGEAL PAPILLOMAS. NUCLEAR-NUCLEOLAR MEAN AREAL DIAMETER RATIO

		II Quartile	III Quartile
Benign:	Case 1	5.6 : 1	4.9 : 1
»	2	4.6 : 1	4.2 : 1
»	3	5.1 : 1	4.6 : 1

		Malign. area	»Benign» area	Malign. area	»Benign» area
Malignant:	Case 4	5.8 : 1	6.9 : 1	5.5 : 1	6.3 : 1
»	5	4.8 : 1	5.8 : 1	3.9 : 1	4.6 :1
»	6	5.7 : 1	5.9 : 1	3.9 : 1	3.6 : 1
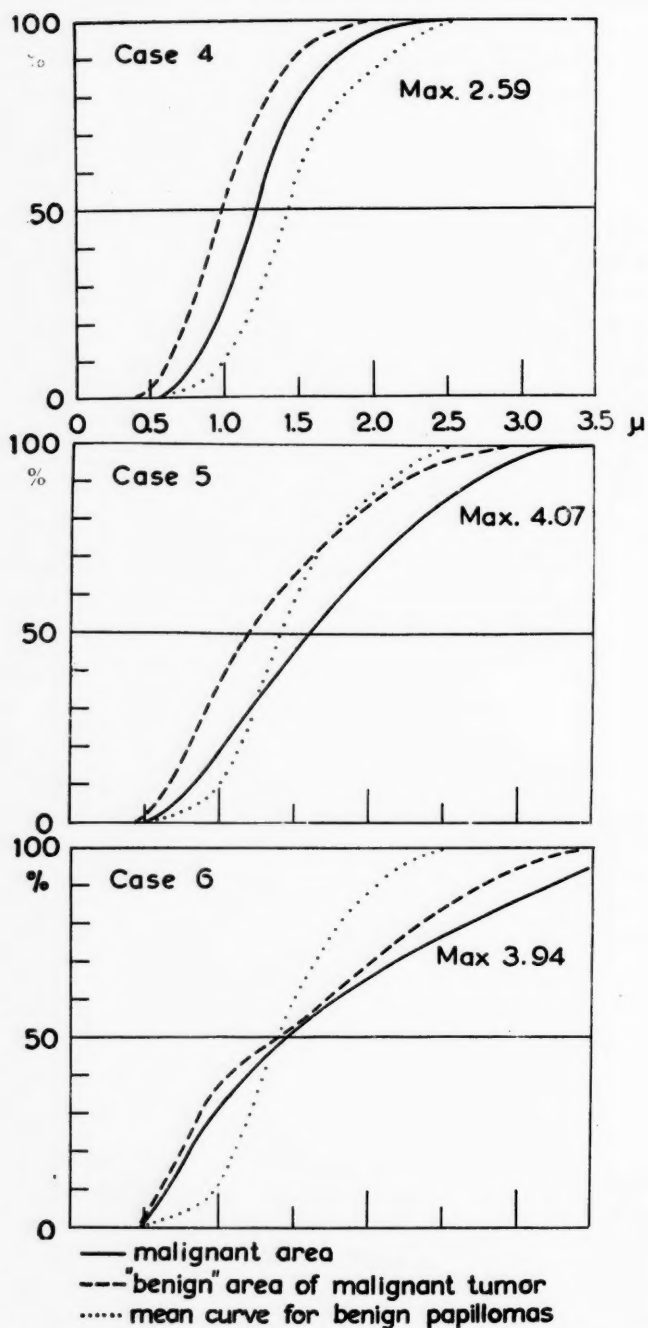
Fig. 2. — Malignant laryngeal papillomas. Cumulative distribution curves of mean areal diameters of nucleoli, malignant and »benign» area. Mean maximal diameters of nucleoli.

DISCUSSION AND CONCLUSIONS

The number of nucleoli per nucleus was not in our series greater in the malignant than in the benign cases, and in two of the malignant preparations the average number of nucleoli per nucleus was even greater in the »benign» area than in the malignant area. These results differ entirely from those obtained by Long and Taylor in studying gynecological tumours. The divergence of the results can be due only in part to differences in the technique used: they must be due essentially to biological differences between the tumours concerned.

Figure 1 indicates that the mean areal diameters of the nucleoli were largely similar in the three benign cases. The curves are uniform in shape and, in the area of the larger diameters, they very nearly coincide. The curves rise steeply, *i.e.* the greater part of the nucleoli were of about the same order of magnitude. The mean maximal diameter, likewise, is approximately the same in all three cases.

The curves for the malignant cases are not nearly as uniform. In two of the cases, 5 and 6, the curve increases slowly in height and the value for the mean maximal diameter is high — these criteria suggest a large variability of nucleolar size. The third malignant case, 4, presents curves differing completely from the other two, although the histological picture was otherwise definitely malignant; these curves bear more resemblance to those seen in the benign cases but show an even steeper rise, thus indicating still greater uniformity of nucleolar size. In agreement with this, the mean maximal diameter is smaller than in any other case. It may be mentioned that when counts of mitoses were earlier made on the same material (1) this very case showed an exceptionally high mitotic rate, 19.7 in 1,000 cells, the highest ever noted by us in papillomatous tumours of the larynx.

When admitted for treatment, the patient in case 1 had a large tumour with metastatic involvement of regional lymph nodes, and according to the past history, the tumour had rapidly increased in size. In case 5 the tumour developed slowly in the course of several years, and in case 6 the tumour was quite small and appeared clinically innocent.

Thus, different types of nucleolar variability may be observed

in malignant papilloma of the larynx: the nucleolar diameter may
vary greatly and very large nucleoli may be present, but on the
other hand, there may be nucleoli which are smaller and of even
more uniform size than in benign papillomas. It is worth noticing
especially that the latter finding, which is probably not usual in
cases of malignant laryngeal papilloma, was made in the one of
the three malignant cases which was by far the most malignant
clinically. It would seem that the two phenomena referred to
above should be regarded as signs of a disturbance in the biological
equilibrium, which — judging from the uniform curves — was
much better preserved in our benign cases.

It is of particular interest to compare the curves obtained for
the malignant and the »benign» areas in the malignant cases. The
curves for the »benign» areas would be expected to resemble those
found in benign papilloma in view of the otherwise great similarity
of histological appearance. In studying the mitotic rate in malignant
papilloma, we found that in these »benign» areas of the malignant
papillomas the mitotic rate corresponded to the figures determined
for the benign papillomas. The curves in figure 2, demonstrate the
surprising fact that the variations in nucleolar size in the »benign»
areas were almost similar to those in the malignant areas: the
curves are on the whole parallel. The observation, of course, is
extremely interesting as it indicates that the local benignancy was
only apparent. Experience has shown that biopsies from malignant
papillomas may now and again result in a specimen which, by
ordinary histological methods, must be classified as benign. If
this observation regarding the nucleoli in apparently benign areas
of malignant papillomas can be further corroborated, this would
no doubt signify increased possibilities of correct histological classi-
fication of preparations from malignant laryngeal papillomas.

Table 2 shows that the nuclear-nucleolar mean areal diameter
ratio, assessed on the basis of the second quartiles, gave on the
whole fairly similar results in the benign and in the malignant
cases. But within the range of the larger nuclei and nucleoli, re-
presented in the table by the third quartiles, the majority of the
quotients showed fairly distinct divergences from the figures
obtained in the benign cases, and this applies to both the »benign»
and the malignant areas of the malignant specimens. In case 4 the
ratio is greater, in cases 5 and 6 smaller, than in the benign cases —

which suggests that in the malignant cases the nucleoli were patho-
logical to a higher degree than the nuclei.

It thus seems that when evaluating whether a laryngeal papil-
loma is malignant or not, nucleolar measurement gives more reliable
results than nuclear measurement. Both these measurements are
necessary, however, since determination of the nuclear-nucleolar
ratio appears to be of value. The measurements take much time
and thus the method is scarcely suitable as a matter of routine
except where there are difficulties of interpretation.

SUMMARY

Measurement of nuclei and nucleoli was carried out on 3 benign
and 3 malignant papillomas of the larynx. In the malignant cases,
the areas that were apparently benign on the basis of the general
histological picture were measured separately.

The number of nucleoli per nucleus was much the same in the
malignant as in the benign cases.

The results of nucleolar measurements are presented in the
form of cumulative distribution curves. These are markedly similar
in the benign cases. The curves rise abruptly, indicating that the
majority of the nucleoli were of about the same order of magnitude.

In the malignant cases the curves present great variability.
In two of them there were large variations in nucleolar size. In
the third case, which was clinically the most malignant one and
showed an exceptionally high mitotic rate, the nucleoli were small
showing even less variation in size than the benign cases. The
»benign» areas in the malignant papillomas presented changes in
nucleolar size resembling those seen in the malignant areas.

The nuclear-nucleolar ratio indicated that the difference be-
tween the malignant and the benign cases were more distinctive
in the nucleoli than in the nuclei. The ratio should be calculated
within the range of the larger nuclei and nucleoli, for example by
utilization of the third quartiles: otherwise the results will not be
informative.

Nuclear and nucleolar measurements are to be considered of
value in disclosing malignancy of laryngeal papillomas. The
method is time-consuming and thus its practical unsefulness seems
to be restricted to cases which are difficult to evaluate and interpret.

REFERENCES

1. Björk, H., and Hakosalo, J.: Acta path. et microbiol. Scandinav. In the press.
2. Björk, H., and Teir, H.: Acta Otolaryng. 1957:47:95.
3. Brofeldt, S. A.: Arb. path. Inst. Hels. 1928:5:34.
4. Castrén, H.: Arb. path. Inst. Hels. 1926:4:240.
5. Cowdry, E. V.: Cancer Cells. W. B. Saunders Co, Philadelphia &London 1955.
6. Guttman, P. H., and Halpern, S.: Am. J. Cancer 1935:25:802.
7. Hoffmann, J. G.: The Size and Growth of Tissue Cells. C. C. Thomas, Springfield & Illinois, U.S.A. 1953.
8. Klossner, A. R.: Arb. path. Inst. Hels. 1931:6:81.
9. Lindberg, K.: Arb. path. Inst. Hels. 1935:8:225.
10. Long, Margaret E., and Taylor, H. C.: Ann. N. Y. Acad. Sc. 1956: 63:1095.
11. MacCarty, W. C.: Am. J. Cancer 1936:26:529.
12. Saxén, A.: Arb. path. Inst. Hels. 1926:4:1.
13. Snellman, A.: Arb. path. Inst. Hels. 1931:6:243.
14. Stenius, F.: Arb. path. Inst. Hels. 1925:3:27.

MITOTIC RATE AND COLCHICINE SENSITIVITY OF THE EPITHELIUM OF THE ESOPHAGUS, TRACHEA AND BRONCHI OF THE WHITE MOUSE[1]

by

HARRY BJÖRK

Organs and tissues have been divided into three groups according to the division potential of cells at various ages, *i.e.* the perennial, stable, and labile elements. In the labile organs, constant physiological cell multiplication occurs, and mitoses are found in them throughout life. Some of these organs, for example the skin and the intestines, have been the subject of numerous studies of the normal mitotic rate and the mitotic reaction caused by various so-called karyoklastics, of which colchicine has been most extensively used. The interest aroused by such studies is based essentially on an observation reported by Bizzozero and Vassale in 1887 (2), namely, that primary cancer is most common in organs of the labile group.

Mitotic phenomena have been found to vary considerably from one organ to another, with resulting differences in the respective mitotic rates. Blumenfeld (3) noted differences in the mitotic activity at various times of the day in the epidermis, the renal cortex, and the submaxillary gland of the white rat. Widner, Storer and Lushbaugh (13), in studying rats and mice, found marked differences in the time taken by mitosis and especially in the intermitotic time in the myelocytic series, the jejunum, the

erythrocytic series, the ovary, the epidermis, the lymph nodes, and the adrenal. To obtain a manysided and correct opinion regarding the mitotic phenomena, it is therefore important to study as many organs as possible under various experimental conditions.

The use of mitotic poisons has increased our possibilities of studying mitotic phenomena. The mode of action of these poisons has been much discussed. According to Ludford (8), colchicine causes arrest of mitoses resulting in a typical reaction with numerous mitoses at metaphase. Analogously, Brues (4) stated that the number of colchicine metaphases present in a tissue could be used as an index of its mitotic activity. Starting from this view, Bertalanffy and Leblond (1), on the basis of their observations on rats, computed the percentage of dividing cells per day in the alveolar lung of the white mouse by counting the metaphases six hours after injection of 0.2 mg. of colchicine per 100 gm. of body weight and multiplying the result by four. Dustin (6), however, expressed the view that colchicine not only arrests but also stimulates mitotic activity. In this opinion he was supported by Teir, who was able to produce in adult rats a large amount of mitosis in the external orbital gland (10), and in the liver (11) which normally show mitoses extremely seldom.

Thus, the opinions regarding the effect of colchicine have been controversial. Although studies with this chemical, as well as with other karyoklastics, have been of importance, caution should be exercised in evaluating the normal mitotic phenomena in an organ on the basis of experiments with karyoklastic substances.

To the author's knowledge, studies of mitotic phenomena in the esophagus, trachea and bronchi have not been previously presented in the literature. Such studies, however, seem to be of interest. The squamous epithelium of the esophagus and the respiratory epithelium of the lower respiratory tract are interesting subjects for comparative studies. In addition, we know that the esophagus and trachea in mice are extremely seldom the sites of tumours, whereas certain strains of this species show a very high incidence of pulmonary tumours of the type adenoma-adenocarcinoma arising from the lining cells of the bronchi and the alveoli (5).

For these reasons it seemed of interest to study the mitotic phenomena in the esophagus, trachea and bronchi, and the albino mouse was chosen for the experiments.

METHODS

The experimental mice used did not belong to any strain with a known tendency to pulmonary tumours. The animals were 2 months old and weighed 20—25 gm.

Forty-five mice were used; 15 of these received no colchicine and served as controls for determination of the normal mitotic rate. In the colchicine experiments, 15 animals were injected subcutaneously with a dose of 0.06 mg. and 15 animals with 0.09 mg. at the same time of the day. The times of exposure to colchicine were 5, 11 and 29 hours, after which periods the animals were decapitated in batches of five. Thus each subgroup of both the untreated and the colchicinetreated group consisted of 5 animals.

The thorax was opened immediately on decapitation. The trachea and esophagus were removed in one piece, in such a manner that both the pipes lay alongside each other. In addition, all four lobes of the right lung were removed for examination.

For fixation, Bouin's solution was used. The preparations were sectioned at 5 μ and stained with hemalum-eosin. The esophagus and trachea were sectioned transversely so that both organs came to be visualized as two circles side by side, which facilitated the counts of mitoses. In the lungs, the counts were done on small bronchi and bronchioli. The number of mitoses was calculated per 3,000 epithelial cells in the various organs of each experimental animal. The counts were done »blindly», *i.e.* the observer — the author himself — was not aware of which preparation he was studying at any given time.

RESULTS

Data on the counts of mitoses are presented in tables 1 and 2. Table 1 shows that one of the subgroups consisted of only 4 animals: colchicine treatment of one of the mice had been accidentally omitted. This was clearly indicated by the unchanged general condition of this mouse, with complete absence of any mitotic response in all three organs studied.

Study of the reactions to colchicine showed that all the mitoses with scarcely any exceptions were typical of this mitotic poison, as illustrated by figures 1, 2 and 3.

TABLE 1

AVERAGE FREQUENCY OF MITOSES PER 1000 EPITHELIAL CELLS OF THE ESOPHAGUS,
TRACHEA AND BRONCHI IN UNTREATED AND COLCHICINE-TREATED MICE

Group	Number of Animals	Sub-groups		
		Killed 19. 1. 57		Killed 20. 1. 57
		3—4 p.m.	9—10 p.m.	3—4 p.m.
I Untreated	15			
Esophagus		4.4	5.9	3.2
Trachea		1.2	0.9	0.9
Bronchi		0.7	4.3	2.5
II Colchicine 0.06 19. 1. 57, 10—11 p.m.	14	a)	b)	c)
Esophagus		41.3	35.9	30.4 [1]
Trachea		25.7	16.0	9.0 [1]
Bronchi		1.2	26.2	6.0 [1]
III Colchicine 0.09 19. 1. 57, 10—11 p.m.	15			
Esophagus		17.6	36.2	47.9
Trachea		15.3	15.8	13.7
Bronchi		1.5	14.3	7.1

a) Time of exposure 5 hours
b) »   »   »   11   »
c) »   »   »   29   »

[1] This sub-group consists of 4 animals, all other of 5 animals.

TABLE 2

DISTRIBUTION OF UNTREATED AND COLCHICINE-TREATED MICE ACCORDING TO
MITOTIC FREQUENCY PER 1000 EPITHELIAL CELLS

Group	Number of Mitoses per 1000 Cells						Total	Average frequency of mitoses per 1000 cells
	0—4	5—8	9—19	20—39	40—59	60—		
I Untreated								
Esophagus	8	7	—	—	—	—	15	4.5
Trachea	15	—	—	—	—	—	15	1.0
Bronchi ..	12	3	—	—	—	—	15	2.5
II—III Colchi- cine-treated								
Esophagus	—	—	7	14	4	4	29	35.1
Trachea	6	4	10	7	1	1	29	16.2
Bronchi ..	15	3	7	2	2	—	29	9.5

Fig. 1. — Photomicrograph showing arrested mitoses in basal layer of esophagus 5 hours after injection of colchicine 0.06 mg. × 510.
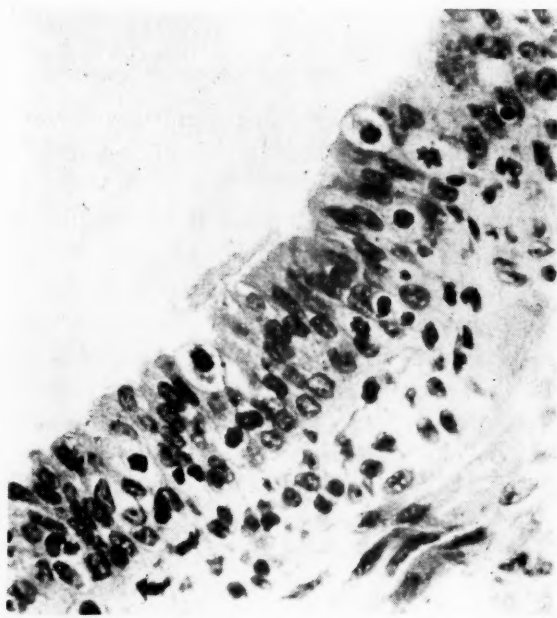
Fig. 2. — Photomicrograph of dorsal wall of trachea: arrested mitoses in different depts of epithelium. × 510.
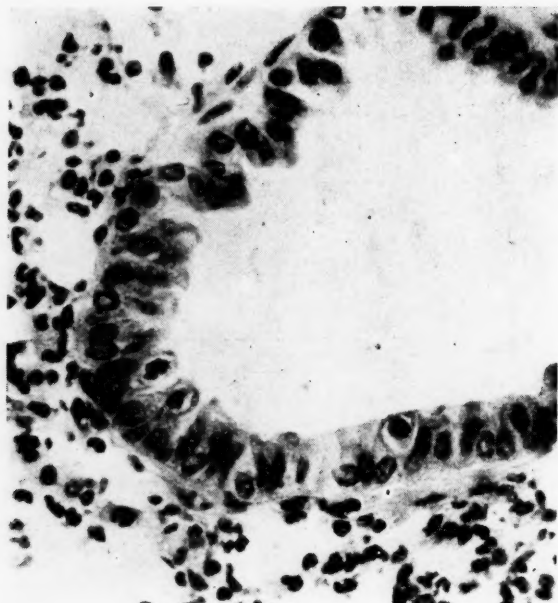
Fig. 3. — Photomicrograph of small bronchus 11 hours after injection of colchicine 0.06 mg: cells in arrested mitosis dislodged towards lumen. × 510.

In the trachea mitoses occurred especially in the dorsal membranous wall where the epithelium is higher than elsewhere and thrown into longitudinal folds.

The mitotic rates in the bronchi presented wide variations not only from one subject to another but also in adjacent bronchi of the same lobe.

The great majority of the mitoses in the esophageal mucous membrane were located in the basal cell layer (fig. 1). In the dorsal membranous wall of the trachea mitoses occurred at different depths in the epithelium (fig. 2). In the small bronchi and bronchioli the cells in mitosis were frequently dislodged from the single row of cells towards the lumen (fig. 3).

The statistical variance analysis of the data was done by Jaakko Kihlberg, M.A.

DISCUSSION AND CONCLUSIONS

The esophagus, trachea and bronchi of 2-month-old mice show a distinct, normal mitotic activity. The mitotic rate is higher in the esophagus (mean 4.5 per 1,000 cells) than in the trachea (1.0/1,000) and in the bronchi (2.5/1,000). The fact that the esophagus in mice is extremely seldom the site of a tumour illustrates that active regeneration in itself is not a factor predisposing to tumour formation.

From the histological specimens it was readily recognizable that regeneration in the organs studied must in part be attributed to loss of cells by desquamation from the surface. In the esophagus the desquamation was particularly intense, in the trachea and bronchi less pronounced but clear enough. Storey and Leblond (9) have interpreted the mitotic activity in the skin as being a consequence of a similar desquamation, and Bertalanffy and Leblond (1) ascribed the active regeneration in the alveoli to the same cause.

Both the normal cell division and the mitotic process following colchicine injection indicate that regeneration in the esophageal mucous membrane and in the respiratory epithelium take place in different epithelial layers. As in the skin (9), cell division in the mucous membrane of the esophagus occurs chiefly in the basal cell layer, while the renewal in the epithelium of the respiratory mucous membrane occurs at various depths in places where this epithelium is stratified. The cause of the displacement of mitotically active cells in the small bronchi and bronchioli in direction towards the lumen seems to be that the dividing cell is swollen and cannot retain its place in the row of cells during mitosis.

In the non-treated as well as the colchicine-treated animals a striking variation was observed in the mitotic rate in the lungs even in adjacent bronchi and bronchioli within one and the same lobe. The explanation of this phenomenon may be that the anatomy and physiological function of the lungs are more complicated than those of the esophagus and trachea. It is therefore understandable that regeneration may differ in different portions of the lungs. Naturally, it is difficult to determine whether this »restlessness» and unbalance in the bronchial and bronchiolar epithelium is causally connected with the predisposition to pulmonary tumours observed in certain strains of mice.

There was no statistically significant difference in the normal mitotic rates between the mice decapitated at 3—4 p.m. and those decapitated at 9—10 p.m. The bronchi, however, showed a certain statistically verifiable tendency to higher mitotic rates before midnight.

All the three organs studied showed a well marked sensitivity to colchicine administered in doses of 0.06 and 0.09 mg. Before starting the experiments under review, we tried the doses 0.01 and 0.02 mg. Analogously to previous observations, we found that these low doses did not cause any distinct reaction in the esophagus, trachea or bronchi. Bertalanffy and Leblond (1) noted that 0.10 mg. of colchicine per hundred gram of body weight is adequate for arresting mitosis in the lung of the rat, whereas 0.20 mg. per hundred gram of body weight is required for the same reaction in the alveoli of mice.

The doses 0.06 and 0.09 mg. caused on the whole a uniform reaction, which was not, however, similar in all the three organs studied. Curiously enough, the squamous epithelium in the esophagus and the respiratory epithelium in the trachea responded essentially in a similar manner: the reaction was already definitely demonstrable 5 hours after injection, and after an exposure period of 29 hours it persisted, being still pronounced. An interesting detail may be noticed. The esophageal reaction at 5 hours after injection was considerably weaker after the larger dose of colchicine than after the smaller dose, but later it became intense. The observation is in accord with Lettré and Krapp's (7) description of the effect of colchicine. They state that the primary effect in the case of large doses consists in prevention of the onset of division (Hemmung des Teilungsbeginnes).

The reaction to colchicine shown by the bronchi and bronchioli diverges greatly from the corresponding phenomenon in the esophagus and the trachea. As a whole, the reaction was weaker. The most interesting feature was the development of the mitotic rate from one observation interval to the next, and this applies to both doses used. Five hours after injection there was no reaction whatever; after the 11 hour exposure period the reaction was distinct; after 29 hours the reaction had largely disappeared. The reaction was considerably stronger after the smaller dose (26.2/1,000) than after the larger dose (14.3/1,000). The result is in

agreement with an observation made by Teir, Kiljunen and Putkonen (12) regarding the effect of tissue extract as a mitosis-stimulating agent: when very large doses were used, there was no mitotic response whatever.

On the basis of the data here presented, the esophagus, trachea and bronchi are suitable for studies of the effect of mitotic poisons. The reaction is very clear. It is remarkable that the mitotic response of the respiratory epithelium in the trachea is practically similar to that of the squamous epithelium in the esophagus, but completely different from that seen in the respiratory epithelium of the bronchi and bronchioli.

A 0.09 mg. dose, subcutaneously injected, seems to be the maximal one in colchicine experiments on mice, if the duration of the experiments is one day. In addition to the 15 animals included in group III of this series, four further animals were given 0.09 mg. of colchicine, having in mind possible deaths during the experiments. At 29 hours after injection one of these mice had died, and the remaining three died within the next 10 hours.

SUMMARY

Studies of mitoses in the esophagus, trachea and bronchi were carried out on 2-month-old albino mice weighing 20—25 gm. Fifteen animals were used for determination of the normal mitotic rate. Twenty-nine animals were injected with colchicine, the doses being 0.06 and 0.09 mg. subcutaneously. Counts of mitoses were made at 3—4 p.m. and 9—10 p.m. The exposure times were 5, 11 and 29 hours.

All the organs studied showed a distinct normal mitotic activity. The mitotic rate was highest in the esophageal mucous membrane (average 4.5 per 1,000 cells), evidently owing to exceptionally intense desquamation of cells from the surface.

Mitotic regeneration in the mucous membrane of the esophagus occurs chiefly in the basal cell layer. Where the respiratory epithelium of the trachea is stratified, mitoses are found both in the basal and in the more superficial layers.

The lungs show great dissimilarity of mitotic rates in adjacent bronchi and bronchioli. This lack of balance in the epithelium may

perhaps be connected with the predisposition to pulmonary tumours found in certain strains of mice.

Colchicine injected subcutaneously in doses of 0.01 and 0.02 mg. did not cause any distinct reaction in the organs concerned, but doses of 0.06 and 0.09 mg. were definitely effective.

The squamous epithelium in the esophagus and the respiratory epithelium of the trachea responded uniformly to colchicine: the effect was already distinctly observed 5 hours after injection, and it persisted and was still well marked after the 29 hour exposure period. The reaction in the small bronchi and bronchioli was of entirely different type: 5 hours after injection there was no reaction whatever as yet; after the 11 hour exposure period it was distinct; after 29 hours the reaction had largely subsided.

A 0.09 mg. dose of colchicine injected subcutaneously into mice weighing 20—25 gm. seems to be the largest possible in experiments of one day's duration. Four additional experimental mice were given this dose and they all died during the second day after injection.

REFERENCES

1. BERTALANFFY, F. D., and LEBLOND, C. P.: Anat. Rec. 1953:*115:*515.
2. BIZZOZERO, G., and VASSALE, G.: Virchows Arch. 1887:*110:*155.
3. BLUMENFELD, M. D.: Arch. Pathol. 1942:*33:*770.
4. BRUES, A. M.: J. Physiol. 1936:*86:*63.
5. CLOUDMAN, A. M.: Biology of the Laboratory Mouse. Blakiston Co, Philadelphia 1941.
6. DUSTIN, A.-P.: Bull. Acad. Méd. Belg. 1934:*14:*487.
7. LETTRÉ, H., and KRAPP, R.: Quoted by LETTRÉ: Ergebn. Physiol. 1950:*46:*379.
8. LUDFORD, R. J.: Arch. exper. Zellforsch. 1936:*18:*411.
9. STOREY, W. F., and LEBLOND, C. P.: Ann. N.Y. Acad. Sc. 1951:*53:*537.
10. TEIR, H.: Acta path. et microbiol. Scandinav. 1944:Suppl. 51.
11. TEIR, H.: Acta path. et microbiol. Scandinav. 1948:*25:*45.
12. TEIR, H., KILJUNEN, AINO, and PUTKONEN, T.: Ann. Chir. et Gynaec. Fenn. 1951:*40:*61.
13. VIDNER, W. R., STORER, J. B., and LUSHBAUGH, C. C.: Cancer Res. 1951:*11:*877.

THE WEIGHTS OF THE BRAIN, HEART, LUNGS, LIVER, AND KIDNEYS OF THE ALBINO RAT AND THEIR PERCENTAGES OF BODY WEIGHT DURING GROWTH, ESPECIALLY IN THE FIRST THREE WEEKS OF LIFE

by

PENTTI JOKELAINEN and PAAVO MÄKELÄ

Because of its many favourable properties, the albino rat is employed as an experimental animal in a most varied range of studies. In connection with our dehydration studies on newborn albino rats we observed from our fairly extensive normal control material the weights of the brain, heart, lungs, liver and kidneys and their ratio to total body weight. Special attention was devoted to the first few postnatal days. As no information is available in the literature on the weights of certain organs of rats in this age period, this paper may help to fill the gap. For the sake of comparison, the weights and percentages of the corresponding human organs are also given.

MATERIAL AND METHODS

The total body weights of 393 and the weights of certain organs of 89 rats of Spraque-Dawley (S-D) race were determined. The animals were divided into series according to age, as follows: 0—4, 8, 11, 16 and 20 days. Up to the series of 16-day old rats the series were made up from a minimum of four different litters, and their ages were determined to an accuracy of a couple of hours. Immediately after preparation, the brain, heart, lungs, liver and both kidneys together were weighed to an accuracy of 1 mg. The potential error in determining the body weight of the living rats was 0.1 g.

RESULTS

The values quoted as birth weights, on which the calculation of the multiplications of body weight is based, differ considerably. Depending on the moment of weighing, the first meal already caused a difference of approx. 0.5 g. In addition, Farris and Griffith (1), have listed a number of other factors affecting birth weight. As the birth weights of newborn, full-term young rats varied, the total weights obviously differ in later stages of growth as well. Similarly the weights of all the different organs show relative variations. As the material employed in the investigation was fairly large and heterogeneous, the mean weights of the organs can be considered fairly reliable without mathematical treatment. The same applies to the percentile ratio of organ weight to body weight during the period of growth.

Both the present values and those obtained from various sources to illustrate the development of the body weight of rats indicate that the gain   in total weight per time unit is largely constant in the first month of life (Table 1). The body weight is doubled after the fifth day of life. It is trebled between the 9th and 10th days. On the 16th day of life, up to when we have careful observations on a more numerous material, the body weight is 5.4-fold. According to the literature, it is 30-fold in 5—6 months old rats. The figure for the present controls, adult rats, was no less than 59 times the weight at birth.

Brain. — Fig. 1 and Table 2 show that the greatest changes in the weight of the brain occur in the first two postnatal weeks. In the three days following birth adaptation to extrauterine life seems to disturb growth; the weight gain during this period is from a birth weight of 216 mg to 297 mg. From the third day onwards growth continues, according to the graph, evenly and vigorously up to the sixteenth day, by when a weight of 1168 mg is reached. La Grutta and Cilentto (7) obtained a mean weight of 1320 mg for the brain of rats of 5—6 months of age. The difference from the age of 16 days is only 152 mg.

By the time the body weight has approximately doubled, the brain mass has also doubled. In this way the development of brain and body weights run roughly parallel for just over the first two weeks. After approx. 16 days the growth of the brain is rapidly
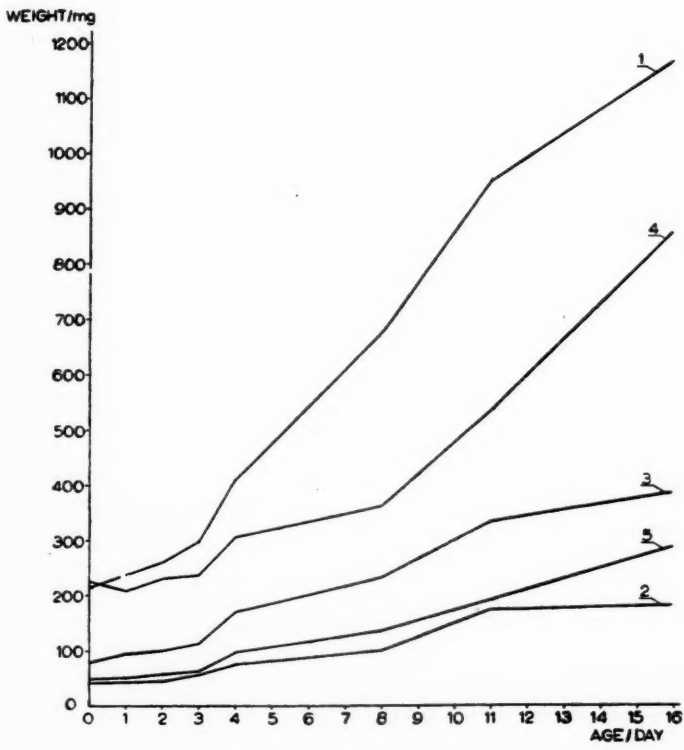
Fig. 1. — The mean weights of the brain (1), heart (2), lungs (3), liver (4) and kidneys (5) of rats during normal growth.

TABLE 1

	Body weight, grammes															
Age, Days	0	1	2	3	4	6	7	8	10	11	16	21	60	70	120—150	Adult
Present material	4.8	5.25	5.55	6.66	8.16	7.68		12.2		21.7	26.2					282.0
Jackson & Lowrey	4.7						9.2					22.3		150.1		
Hamilton-Dewar	5.7		6.6		8.2	11.8		15.5	20.0				176.0			355
McKey Bergman														158.5		
La Grutta & Cilentto														95.0		

Mean body weights of the rat at different phases of the period of growth.

TABLE 2

MEAN WEIGHTS OF ORGANS IN EACH AGE CLASS AND THEIR PERCENTAGE OF TOTAL WEIGHT. MEA
TOTAL WEIGHTS REFERRING TO THE MATERIAL IN BRACKETS ARE ALSO GIVEN IN BRACKETS

Age, Days	No.	Brain		Heart		Lungs		Liver		Kidney		Total Weight
		Mean, mg	%	Mean, mg	%	Mean, mg	%	Mean, mg	%	Mean, mg	%	Mean, g
0	7 (24)	216.1	4.0	41.0	0.7	80.0	1.5	226.9	4.2	49.0	0.9	5.3 (4.8)
1	14 (62)	237.1	4.2	43.0	0.8	96.0	1.7	210.2	3.8	51.4	0.9	5.5 (5.2)
2	10 (46)	259.9	4.3	44.7	0.7	101.4	1.7	231.5	3.9	58.6	1.0	5.9 (5.5)
3	9 (88)	297.0	4.7	57.6	0.9	112.5	1.8	238.2	3.8	64.5	1.0	6.3 (6.6)
4	10 (47)	408.3	4.9	76.6	0.9	171.6	2.1	305.9	3.6	98.1	1.2	8.3 (6.1)
6	2 (6)	429.3	5.6	72.3	0.9	176.3	2.3	238.0	3.1	90.0	1.2	7.6 (7.6)
8	10 (56)	679.2	5.5	101.0	0.8	232.3	1.9	362.0	3.0	135.8	1.2	12.2 (12.7)
11	11 (11)	952.5	4.4	173.9	0.8	332.4	1.5	534.0	2.5	191.0	0.9	21.7 (21.7)
16	11 (45)	1167.9	4.2	182.5	0.6	386.3	1.4	856.2	3.1	288.8	1.0	27.8 (26.2)
5—6 months	8	1320.0	1.4	380.0	0.4	550.0	0.5	3520.0	3.6	790.0	0.8	96.0
Adult	5 (7)	1651.6	0.6	1129.6	0.4	1590.1	0.5	9278.2	3.3	1868.2	0.6	280.0(282.0)

retarded. This is illustrated by the fact that the weight of the brain of an adult rat is only 7.7 times that of a newborn rat.

A closer study of the brain:body weight ratio (Graph 2 and Table 2) shows that from 4.0 per cent at the moment of birth it rises by approx. 14 per cent in the first week of life. At this stage, therefore, the brain has grown slightly faster than the body. By the 11th day of life the percentage has returned to the value at birth. From this time onwards the brain:body ratio decreases continuously; at the approximate age of 6 months the value is 1.4 and in old age 0.6 per cent.

Liver. — The liver, similar in weight at birth (226.9 mg) to the brain, develops more slowly in the first week of growth (Fig. 1 and Table 2). Its weight decreases in the first day of life by approx. 6 per cent, possibly due to the change in physiological conditions. The extrauterine phase of growth proper seems to begin after the third day of life, as with the brain. By the 16th day the weight of the liver is 856.2 mg. Although growth continues slightly more slowly up to adulthood, the rate is relatively vigorous compared with the other organs.
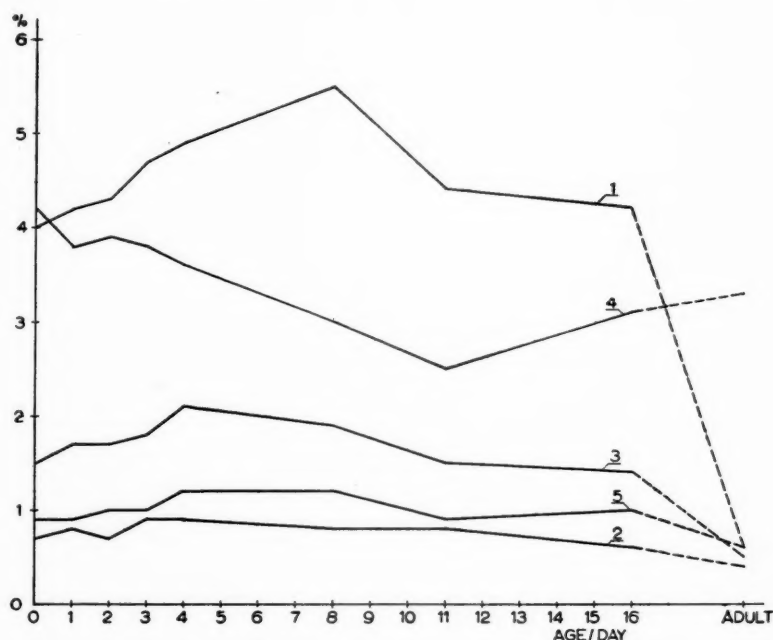
Fig. 2. — The brain (1), heart (2), lungs (3), liver (4) and kidneys (5) of rats as a percentage of the body weight during normal growth.

According to Fig. 1, the weight of the liver doubled between the 9th and 10th days, did not treble itself until the 13th—14th day, and by the 16th day of life had increased 3.8-fold. Compared with the brain, the weight increase of the liver continues still very vigorously after two weeks. As a result, the weight of the liver is 15 times the birth weight somewhat before the age of six months (La Grutta and Cilentto) (7), and 41 times greater in adulthood.

Due to the relatively slow weight gain of the liver in the early days of extrauterine life as compared with body weight, the ratio for this organ drops from the initial value of a good 4 per cent to approx. 2.5 per cent in the first 11 days of postnatal life. From this time onwards, however, the percentage again begins to rise, but remains below 4 in adults.

Heart, Lungs, and Kidneys. — The heart, lungs and kidneys, all with a similar birth weight (heart 41.0 mg, lungs 80.0 mg and kidneys 49.0 mg) constitute a group of their own. In the first postnatal days the weight increase of these organs is approximately

uniform up to the eleventh day. From that age onwards, the growth of the heart and lungs slows down but the kidneys continue to grow vigorously up to adulthood.

The lungs and kidneys double their weight in four days, the heart after the fourth day (Table 2 and Fig. 2). The weight of all these organs is trebled by approximately the ninth day. When the rats are 16 days old, the weight of the kidneys is 5.9-fold, that of the heart 4.4-fold and of the lungs 4.8-fold bigger. By the age of 6 months, the multiplications are so dispersed that the lungs show the figure 6.9, heart 9.3 and kidneys no less than 16. The weight of the heart of an adult rat is 27 times its birth weight, that of the lungs 20 and that of the kidneys no less than 38 times the weight at birth.

Due to the relatively slow growth of the lungs, their percentage of total body weight decreases during the period of growth from 1.5 to nearly 0.5 per cent (Table 2 and Fig. 2). As for the kidneys, the percentage remains fairly steady, declining from 0.9 to 0.6 only. The heart falls between the two, and the percentile change during the whole growth period is somewhat smaller than that of the kidneys. However, the difference between the percentages at birth and in adulthood is the same, 0.3 per cent.

DISCUSSION

The results show that the brain differs considerably in total growth from the other organs studied. The change in total weight is small, as is indicated by the multiplicator, 7.7. But growth is very vigorous in the first 16 days, by which age the weight of the brain is 5.4 times its weight at birth. The difference between these figures is 2.3. It may be concluded that in the remaining period of growth, approximately a whole year, the increase in the weight of the brain is only minimal.

A similar development is noted in the growth of the human brain in relation to the whole body. The weight at birth is trebled in the first three years in boys (10); at 20 years of age the weight of the brain is only 3.5 times its birth weight.

In relation to body weight, the human brain is at its maximum of 15 per cent roughly in the 7th month of pregnancy (9), and then begins to decrease. At the moment of birth the weight of the brain, according to various sources, amounts to approx. 10 per cent of

the total weight, and the figure for adults is by Cuvier (2) 4.5—2.8 per cent, by Tiedeman (2) 2.4—2.38 per cent. The maximum for rats seems to occur after birth at the approximate age of one week, when the brain:body ratio is about 5.6 After completed growth the weight of the ratio remains below one-third of the figure for man.

The two important organs of metabolism, the liver and the kidneys, seem to grow very vigorously in the rat. This is clearly illustrated by the great change in their total weight in the present material; the weight of the former increased 41 and of the latter 38 times. The liver obviously encounters growing difficulties for a good week after birth. This is seen when the multiplications are compared. It may be explicable in part from the immaturity of the liver in both the histological and the physiological, functional sense. Not until 16 days after birth does it catch up with the other organs, and then continues to grow approximately parallel with the kidneys. Up to approximately six months of age the brain, heart and lungs definitely lag behind the growth shown by the multiplications of the liver and the kidneys.

It is even remarkable that the human liver and kidneys also show features similar to the above in the way their weight changes during growth. The weight of the child's liver approximately doubles at the age of 7—12 months, that of the kidneys increases threefold (14). Not until after the 2nd—3rd year of age is the growth of the liver accelerated; according to Gundobin (2), it is at its most vigorous at about the age of sexual maturity. At this time both the liver and the kidneys weigh 11 times their weight at birth.

At the moment of birth the rat liver is 4.2 per cent of the body weight. This value falls between the limits quoted for man (10). Both with man and with rat the ratio decreases slowly during growth For adult rats it is over, and for men under 3 per cent.

The initial kidney: body weight ratios are 0.9—1.0 for rat, 0.7—0.8 for man. In the course of growth, both decline by 0.2. For the rat, the difference is non-existent in the first three months (5), and by six months the difference is 0.1 according to values quoted by La Grutta & Cilentto (7). Hence the increase in kidney weight is practically directly proportional to the increase in total body weight.

As was seen in the discussion of the results, the weights of the lungs and heart, and also of the kidneys doubled and trebled at about the same time, at the age of 4 and 9 days. The body weight increases proportionately. It should be noted that the increase in the weight of the heart and lungs after 16 days decreases as compared with that in the weight of the liver and kidneys and total weight. By the time the growth proper of the heart and the lungs can be considered to have stopped, the former is 27 times and the latter 20 times its weight at birth.

Differently from the corresponding organs of the rat, the human heart and lungs show a different rate of growth by the time their weights have doubled. For the lungs this occurs in approx. the 5th—6th month and for the heart not until after the 8th month (10, 14). By the time its weight has grown 3-fold at the age of 2—3 years the heart lags 1—2 years behind the lungs in growth. By the age of sexual maturity, however, they are at the same level, approx. 11 times their weight at birth. Hence the development in the weight of these organs in man is much more marked than in the rat.

Similarly to the liver and kidney, the heart and lung ratios to total weight also decline within fairly narrow limits compared with the brain. Approximately the same phenomenon is discernible in man too. The percentage of the heart at birth is 0.7 for rat and approx. 0.68 for man. By adult age the percentages have changed to 0.4 for rat and 0.54 for man. In the lungs the change is greater, from 1.5 to 0.5 for rat and from 2 to 1 for man.

Several similarities were observed between rat and man in the increase in weight of the organs discussed. With man the multiplications seem to remain at a maximum of one-third of the level for the rat. As to chronology, man reaches the level of a 2—3-week old rat at approximately three years. A life of 3 years for the rat is considered to equal approx. a 90 year span for man.

SUMMARY

Using a series of 393 rats, the development of the weight of the brain, heart, lungs, liver and kidneys during growth and in relation to total weight was systematically studied. The increase in the weight of the brain was notably retarded after the 16th day

of age, but the other organs continued to grow fairly vigorously. Special mention must be made in this connection of the kidneys and the liver. The change in total weight is also remarkable, equalling about 3 times that in man in 1/20th of the time.

REFERENCES

1. FARRIS and GRIFFITH: The Rat in Laboratory Investigation. London 1942.
2. GUNDOBIN, N. P.: Die Besonderheiten des Kinderalters. Berlin 1912.
3. HAMILTON, B., and DEWAR, M. M.: Growth 1938:2:13—23.
4. JACKSON, C. M., and LOWREY, L. G.: Anat. Rec. 1912:6:449—72.
5. KITTELSON, J. A.: Anat. Rec. 1919:17:281—97.
6. LOWREY, L. G.: Anat. Rec. 1913:7:143—68.
7. LA GRUTTA, G., and CILENTTO, A.: Arch. Sci. Biol. 1954:38:366—376.
8. MACKAY, and BERGMAN, H. C.: J. Biol. Chem. 1934:105:59—62.
9. POTTER, E. L.: Fetal and Neonatal Death. Chicago-Illinois 1940.
10. RÖSSLE, R., ROULET, F., ASCHOFF, D., ELIAS, H., EPPINGER, H., STERNBERG, C., and WENCKEBACH, K. F.: Pathologie und Klinik in Einzeldarstellungen. 1922.
11. SCHÜTZ, W.: Virchows Arch. 1926:259:349.
12. THOMA: Untersuchungen Über die Grösse und Gewicht der anatomischen Bestandteile des menschlichen Körpers. 1882.
13. TKATSCHENKO: Über die pathologisch-anatomischen Veränderungen der Nieren bei Masern. (Russ.) Petersburg 1903.
14. UOTILA, U.: Über die Organgewichten bei den Finnen. Separatabdruck aus Arbeiten aus den pathologischen Institut der Universität Helsingfors. 1942.
15. VIERORDT, H.: Anatomische, physiologische und physikalische Daten und Tabellen. Jena 1906, 3 Aufl.

THE WATER CONTENT IN NORMAL CONDITIONS OF THE BRAIN, HEART, LUNGS, LIVER, KIDNEYS, AND STRIATED MUSCLES OF THE ALBINO RAT, ESPECIALLY IN THE FIRST DAYS OF LIFE

by

PENTTI JOKELAINEN and PAAVO MÄKELÄ

A lowered water content is characteristic of all living organisms in both extrauterine and intrauterine growth and development; the rate and amount of the reduction is dependent on many factors (12). A closer study of the problem reveals relatively great variations between the organs of one and the same individual during dehydration. This was observed *e.g.* in the investigations by L. G. Lowrey (11) into the total changes in the water content of an albino rat. His and other similar investigations devote no special attention to the water contents of the organs in the early weeks of life. To this end, the present authors have determined the water content of the brain, heart, lungs, liver, kidneys and muscle tissues of young rats in the first days of life.

MATERIAL AND METHODS

The series comprised 88 albino rats of the Sprague-Dawley race, divided into age groups of 0—4, 8, 11 and 16 days. In addition, for the sake of correlation, the moisture content was measured of the organs of adult rats (age 1—3 years). Each series comprised some 10 rats, made up from a minimum of four litters. The age

of the young up to 16 days was determined to an accuracy of a few hours from the moment of birth. Almost all the newborn rats were successfully isolated from their mothers before lactation. On reaching the age required for the study the rats were killed by decapitation and the blood was drained off. Immediately after this the brain, heart, lungs, liver and kidneys were removed, together with a piece of some 100 mg of the muscles of the back. The fresh weights were determined to an accuracy of 0.1 mg in cups weighing approx. 1.5 g and fitted with lids; the cups were specially made for weighing and drying purposes. The organs and sections were dried in an incubator containing silica gel, at $+84°C$, until the weight was constant. The weighing cups with preparations were frozen in a vacuum desiccator. On repeated weighing the weight losses, and hence moisture differences, were obtained. (There was no differentation by sex.)

Brain. — In the early stages of growth, the brain is one of the organs with the highest water content. For an unsuckled newborn rat the water content of the brain, according to our determinations, was 88.1 per cent (Fig. 1 and Table 1). Apart from the high water content of the brain at the moment of birth, it is remarkable that the developing brain tissue seems to retain its moisture content practically constant up to the eighth day of life, when the water content was still 87.9 per cent. Three days later, when the rats reached an age of 11 days, it was 86.8 per cent. Hence the moisture in the brain tissue declined by 1.1 per cent in a short while. The Fig. shows, further, how the percentile change was progressive from the 11th to the 16th day of life; the difference during this period was 3.8 per cent. In other words, the absolute water content at the age of 16 days was approx. 83.1 per cent. The difference from the 16th to 20th day of life, again, was only 0.88 per cent, and the percentage at 20 days was 82.2.

La Grutta and Cilentto (10), of Italy, in connection with other studies, determined the total water of the brain of four rats aged 5—6 months; the result was 78.73 ± 0.462. Our determination of the total water in the brain of healthy adult rats who had lived in normal conditions showed the brain tissue to have lost more water, as the water content was 77.4 per cent. The total change from birth to adulthood therefore was approx. 11.5 per cent. Donaldson (2) obtained roughly the same result, for his figure was 9.6 per cent.
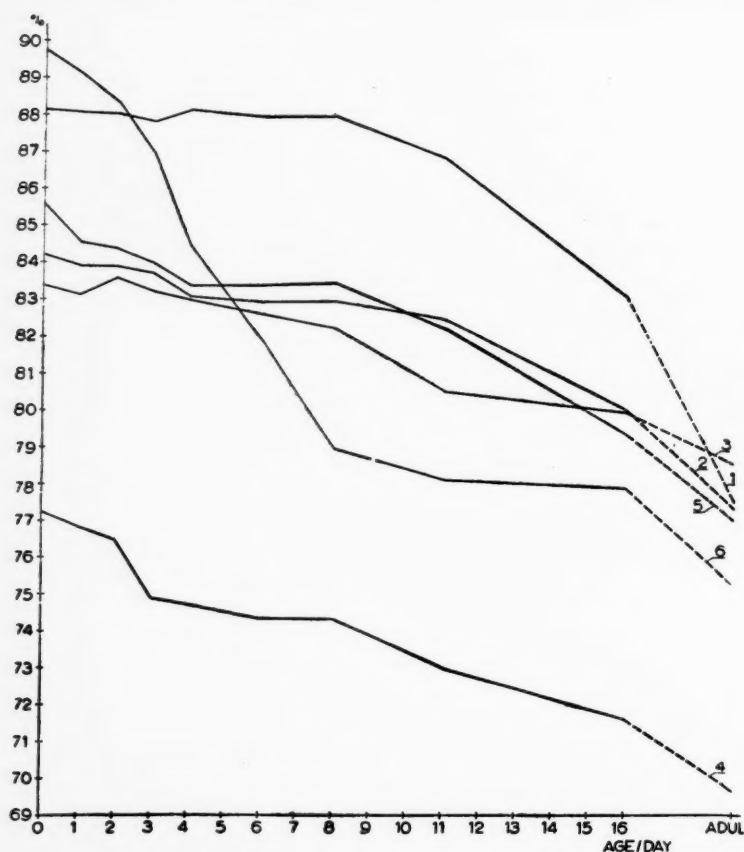
Fig. 1. — The percentage of water in the brain (1), heart (2), lungs (3), liver (4), kidneys (5) and musculature (6) of the albino rat during normal growth.

TABLE 1

THE PERCENTAGE OF WATER AND ITS STANDARD DEVIATIONS IN THE BRAIN, HEART, LUNGS, LIVER, KIDNEYS AND MUSCULATURE OF THE ALBINO RAT DURINGNORMAL GROWTH

Age days	No.	Brain		Heart		Lungs		Liver		Kidneys		Musculat.	
		%	Sd	%	Sd	%	Sd	%	Sd	%	Sd	%	Sd
0	7	88.14	0.24	84.21	0.89	83.37	0.26	77.24	0.71	85.57	0.82	89.73	0.30
1	14	88.06	0.28	83.90	0.60	83.12	0.55	76.85	1.12	84.53	0.70	89.10	0.40
2	10	88.01	0.33	83.69	0.68	83.56	0.69	76.50	0.65	84.38	0.42	88.33	1.10
3	9	87.81	0.29	83.70	0.49	83.20	0.77	74.90	0.71	83.97	0.36	86.98	1.70
4	10	88.10	0.39	83.05	1.20	82.99	0.36	74.73	2.30	83.38	0.63	84.47	1.80
8	10	87.94	0.24	82.94	0.67	82.21	0.37	74.35	0.68	83.45	0.82	78.97	1.40
11	11	86.80	0.30	82.43	0.56	80.52	0.50	73.00	1.20	82.20	0.87	78.10	1.50
16	11	83.08	0.73	80.00	0.38	79.84	0.47	71.60	1.08	79.35	1.68	77.90	1.30
Adult	6	77.55	0.30	77.36	0.52	78.58	0.49	69.68	1.94	77.02	1.12	75.22	0.42

Liver. The other big ·organ, the liver, represents the other extreme as its water content at the moment of birth is only 77.2 per cent. The »physiological» dehydration of the liver begins immediately on birth. In the first three days of life the water content was reduced by 3 per cent. Subsequently the proportion of water decreased evenly. On the sixth day the value was 71.6 per cent, the difference from the birth value being approx. 7.5 per cent. The water content of the liver of the adult rat was 69.7 per cent; hence the change from the age of 16 days, up to nearly a year later, is only approx. 2.5 per cent whereas the change from 0 days is approx. 10 per cent.

Heart, Lungs and Kidneys. — In the group of heart, lungs and kidneys attention is attracted by the relatively great dehydration of the kidneys in the early days. They showed a sharp reduction in water content already in the first day of life, from 85.5 to 84.5 per cent or by approx. 1 per cent. The heart and the lungs lost less water in this period. In the heart, the percentage of water dropped from 84.2 to 83.9 (difference 0.5 per cent) and in the lungs from 83.7 to 83.1 (difference approx. 0.4 per cent). From the second day of life onwards the similarity in the physiological dehydration of these three organs is illustrated by the fact that at the age of 16 days the water content of the organs was between 80.0 and 79.3 per cent. The water content of the kidneys was lowest in adulthood, 77.0 per cent; the change from birth was 10 per cent. The moisture content of the lungs remained at 78.65 per cent. The difference from birth was minimal, 6 per cent. The heart came in between these two extremes, with a percentage of 77.3 and a difference of 8 per cent.

Muscle. — The last in the group of organs studied, the muscle, experiences the greatest changes in moisture content. Right from the beginning the physiological dehydration was very rapid. From the value at birth, 89.7 per cent, there was a drop to 88.3 per cent in two days. After this the dehydration continued at an increasing rate up to the eighth day by when the water content had dropped to 78.8 per cent, the difference from the birth value being 12 per cent. In the remaining growth period the water content was reduced within very narrow limits, the final value being 75.2 per cent. The total change was 16 per cent.

DISCUSSION

Hamilton and Dewar (4) showed in their investigations that the dehydration of the rat's body in normal conditions was more rapid in the first ten postnatal days than in the preceding foetal period. In the next period, from the tenth day onwards, growth in relation to water content continued exactly like that in the last foetal week. Rapid dehydration in the first week of life, according to Hatai (5), is propably due in part to the plentiful increase in adipose tissue. For the sake of comparison with the results obtained for the different tissues, it may be mentioned that at the moment of birth the total water content of the rat varies from 87—88 per cent, according to different authors. At the age of ten days it is approx. 77 per cent and in adult rats 67—68 per cent (1, 11, 4).

A hypothesis has been advanced according to which tissues with a high water content grow more slowly than those poor in water (4). However, it seems that this does not apply to the brain as, in spite of its high water content immediately after birth, it grows very vigorously compared with the other organs (8). As the water content remains at approx. 88 per cent up to the eighth day of life, the difference developing up to the 16th day is nearly 6 per cent. During this period the weight of the brain increases evenly up to the 16th day. As the dry matter percentage in the brain remains unchanged for the first week of life, the weight gain of the brain must be attributed to the more rapid increase of tissue. Taking into account the standard deviation, attention is focused on the relatively small deviation, which remains roughly constant throughout the period of growth.

Compared with the corresponding human development, the total change takes place within identical limits; according to Hungerland (6) it is 89—79 per cent, or a change of approx. 12 per cent. The figures for the rat were 88.1—77.5 per cent, approx. 12 per cent.

As to the liver, a minimal weight gain in the first three postnatal days corresponded to a 3 per cent drop in water content, i.e. the amount of dry matter increased by 9.5 per cent. After this the loss of water was even and as from the 16th day onward greatly

retarded. By contrast a vigorous gain in weight continued throughout the period of growth.

The standard deviations are considerably greater and more variable for the liver than for the brain. This indicates the great lability of the organ. A contributory factor may be its hyperaemic condition and the changes in this condition; for man, for instance, the percentage of blood may amount to nearly 50 per cent (13).

According to Vierordt (14) man at the moment of birth has a water content of 80.55 per cent in the liver; the percentage for adults is 77—68. According to values collected by Hungerland (6) the water content of the newborn is 81 and of adults 74 per cent. Hence the total change is approx. 8.5 per cent with man and 10 per cent with rats.

During the first day of life, the heart, lungs and kidneys show a slight reduction in water content. The weight gain of these organs is slower up to the age of three days. Subsequently the growth is accelerated while the change in water content levels off correspondingly. Between the 8th and 16th day of life the dehydration is most marked among these three organs in the kidneys. Their growth also seems to be intensified in this period. At the age of 16 days, heart, lungs and kidneys all have a very similar moisture content, the kidneys having the lowest value. The moisture content of the lungs remains within fairly narrow limits throughout life, the greatest change taking place in the course of the first postnatal week. The water content of the heart remains throughout the period of growth between that of the kidneys and the lungs. The average daily dehydration is greater in the period from the 8th to 16th day than from birth up to the 8th day.

The values of the heart show a relatively great variation from the standard deviation at different ages; the lungs, by contrast, are especially stable in water content. After the first week, the standard deviation of the kidneys grows, *i.e.* the values show less uniformity.

In man the total change in the water content of the lungs is of the order of 4.5 per cent — from 83 to 79 per cent; with rat the figure was 6 per cent. The moisture content of the human heart drops from 88 to 80 per cent, total change 9 per cent. With rats this is somewhat smaller, 8 per cent (6).

According to Jackson and Lowrey (7), the total weight of the

muscles falls during the first postnatal week of the rats. In the present material, this was when the greater part of the dehydration occurred in the muscles. Subsequently, growth continues evenly up to the tenth week, the decline in water content equalling but a fraction of its previous value.

Evidently as a result of technical difficulties, in part, the percentages indicating the water content of the muscles show the greatest dispersion; the standard deviation remains above 1 apart from the first two days and adulthood.

Hungerland (6) has it that the muscles of the newborn contain 82 per cent of water and those of the adult 74 per cent. Hence the difference between the percentages is approx. 9.5. This difference is in rats 16 per cent, nearly twice as great as in man.

An overall study shows that the weight of the organs increases more slowly in the first three postnatal days. In this period, the organs show a more rapid dehydration. While the loss of weight of the muscles persists throughout the first week of life, the loss of water from the muscles is at its highest during that period. A remarkable exception to this rule is the brain, where the water content remains nearly constant throughout the early period of growth. In addition, it will be noted that in all the organs listed above a large proportion of the dehydration took place in the course of a good fortnight.

SUMMARY

An investigation was made on 88 albino rats to follow the water contents of the brain, heart, lungs, liver, kidneys and muscles as a function of age particularly in the first few postnatal days. In a good fortnight the increase in the percentage of dry matter was so great that during the rest of total growth, in the course of nearly a year, the reduction in water content was minimal. The percentage of muscle dehydration was almost equal within the first week to that of the entire subsequent period of growth. In the brain it only starts after the first week of extrauterine life. The heart and the lungs were more stable as regards moisture content. The liver comes in a special category as it is originally the driest organ of the material. Its dehydration was at its most intense in the first 3—4 days.

REFERENCES

1. Y CONZALES, A. W. A.: Anat. Rec. 1932:52:117—138.
2. DONALDSON, H. H.: Jour. Comp. Neur. 1920:20:119—144.
3. FARRIS, GRIFFITH: The Rat in Laboratory Indestigation. — London 1942.
4. HAMILTON, B., DEWAR, M. M.: Growth 1938:12:13—23.
5. HATAI, S.: Am. J. Anat. 1917:21:23—37.
6. HUNGERLAND, H.: Sonderabdruck aus der »Biologische Daten für Kinderarzt» von Joachim Broch. — Berlin 1954.
7. JACKSON, C. M., LOWREY, L. G.: Anat. Rec. 1912:6:449—472.
8. JOKELAINEN, P., MÄKELÄ, P.: (in press).
9. KITTELSON, J. A.: Anat. Rec. 1920:17:281—297.
10. LA GRUTTA, G., CILENTTO, A.: Arch. Sci. Biol. 1954:38:366—376.
11. LOWREY, L. G.: Anat. Rec. 1913:7:143—168.
12. NEEDHAM, J.: Biochemistry and Morphogenesis. — Cambridge 1951.
13. POTTER, E.: Fetal and Neonatal Death. — Chicago, Illinois 1940.
14. VIERORDT, H.: Anatomische, fysiologische und physikalische Daten und Tabellen. — Jena 1906.

THE EFFECT OF EXPOSURE TO LOW PRESSURE, OF RESTRAINING AND OF INJECTIONS OF ADRENALINE ON THE RATE OF REDUCTION OF METHAEMOGLOBIN IN RABBIT ERYTHROCYTES

by

EEVA JALAVISTO[1] and J. SUNDBERG

It has become evident that increased erythropoietic stimulation of the bone marrow following repeted bleedings (10) or exposure to low oxygen pressure is connected with changes in the chemical composition of blood as shown, among others, by an increased plasma potential (oxidation reduction potential) (5). Furthermore certain findings (1, 2, 0, 4) seem to indicate that blood plasma may become erythropoietically active when incubated preferably under low oxygen pressure in the presence of red cells and reticulocytes, but not otherwise. Obviously some chemical component of the red cells is in this case the source of the stimulating factor present in blood plasma. The metabolism of erythrocytes, therefore, deserves special attention.

Small amounts of methaemoglobin are formed constantly by auto-oxidation of haemoglobin. This would lead to loss of functioning haemoglobin and possibly to disintegration of red cells if it were not opposed by regeneration of haemoglobin through reduction of methaemoglobin. Matthies & al. (8) have shown that the rate of methaemoglobin reduction (MRR) in erythrocytes treated with sodium nitrite is enhanced by repeated bleedings. The

aim of the present paper is to study wether other forms of hypoxic stimulation likewise result in an increased rate of methaemoglobin reduction. Certain unsuspected, transient variations in the MRR during the course of the experiments made it necessary to pay special attention to the handling of the animals and to include experiments with injections of adrenaline, since, as will be shown, the mobilization of adrenaline when the rabbits are restrained, may affect the methaemoglobin reduction rate.

METHODS

The MRR was determined principally according to the method of Künzer (7). 2—4 ml of blood were withdrawn from the marginal ear vein of rabbits in the morning, each time approximately at the same hour. The samples were kept in ice-water until diluted with 1 per cent sodium nitrite solution and incubated during ½ hour at 35 centigrades. The erythrocytes were separated from plasma and were washed 5 times with saline and suspended in a phosphate buffer solution adjusted to pH 7.4 to make 2 ml. 10 mg of glucose were added to this amount of suspension. A drop of the suspension was dissolved in destilled water and the content of methaemoglobin was determined spectrophotometrically at the wavelengths 630 and 577 m μ according to the nomographic method of Hunter (3). After exactly 1 hours incubation in Warburg flasks at 37.1 centigrades under shaking a second sample was similarly analysed. The difference in methaemoglobin concentration between the first and second sample was expressed as percentage of the initial concentration and was taken to represent the MRR. In 10 experiments with injections of adrenaline parallel determinations of MRR after addition of 0.002M iodoacetic acid, were made.

In seven rabbits blood samples were taken on different days twice before and twice following a period of exposure to low pressure (7—8 days, 6 hours a day at 360 mmHg, which regularly induced polycythaemia in the rabbits). In 13 other rabbits experiments with injections of adrenaline were performed as follows: 20 minutes after a first sample of blood, a second one was taken and served as control. Adrenalin 0.25 mg was then injected subcutaneously and a third sample was taken 15 minutes after the injection. A fourth sample was withdrawn 40—45 minutes after the injection. 9 rabbits

were put into an animal holder box forcing the head into the right position by pulling from the ears. The rabbits usually struggled against and the group is called »restrained rabbits». In 10 rabbits (6 of which belonged to the »restrained» as well) the procedure was otherwise the same except that the rabbits were handled with extreme caution and were allowed to sit free on the table (»un-strained» or »control» rabbits).

RESULTS

The results are summarized in table 1. The first colum gives the experimental group of rabbits, the second indicates the mean initial

TABLE 1

Experimental group, rabbits	M R R %		t	P	Signific-ance
	Initial	Increase			
Control	56.3 ± 2.78	— 2.1 ± 1.93	1.09	>0.1	not s.
Hypoxia	54.0 ± 2.85	+10.1 ± 1.72	5.87	<0.001	highly s.
Restraining,					
20 min.	56.6 ± 3.35	+ 6.2 ± 2.25	2.76	<0.05	probably s.
Adrenaline,					
15 min. (250 γ					
s.c.) restrained	63.0 ± 3.76	+ 0.7 ± 2.31	0.3	>0.1	not s.
unstrained	55.0 ± 4.06	+13.2 ± 2.34	5.65	<0.001	highly s.
Adrenaline, 45 min.					
(250 γ s.c.)					
restrained	63.0 ± 3.76	+ 5.8 ± 2.74	2.11	>0.05	not s.
unstrained 1) ..	56.3 ± 2.78	+14.6 ± 3.00	4.96	<0.001	highly s.
2) ..	55.0 ± 4.06	+16.7 ± 2.46	6.80	<0.001	highly s.

MRR in per cent, with its standard error. The third column contains the mean increase (with its standard error) calculated on basis of the individual initial values. The columns 4 and 5 show the values of t and P for an evaluation of the significance of the change (increase) in MRR, which is given in the last column.

As may be seen from the table a highly significant increase in the MRR occured in the hypoxia group after the periode of exposure to low athmospheric pressure, the post-exposure MRR being about 64 per cent whereas the initial mean values of this and other experimental control groups did not on an average exceed 56.6 per

cent. In unstrained rabbits both in the samples taken 15 and those taken 40—45 minutes after the injection of adrenaline, a considerable elevation of the MRR has taken place. In the restrained rabbits, however, no such increase is present, obviously as the elevation of the MRR occured already before the injection of adrenaline; most likely because of a mobilization of adrenaline by the restrain. The increase effected by mere restraining amounts to 6 per cent, which is probably significant. No such increase is seen in the control group between the MRR of the first and second sample the tendency being, on the contrary, that of a decrease. The higher values of MRR in the restrained rabbits may, therefore, be considered as significant (P < 0.01), if the comparison is made between the corresponding samples of controls and of restrained rabbits. The graph in fig. 1 clearly shows the similar effect of adrenaline and restrain.
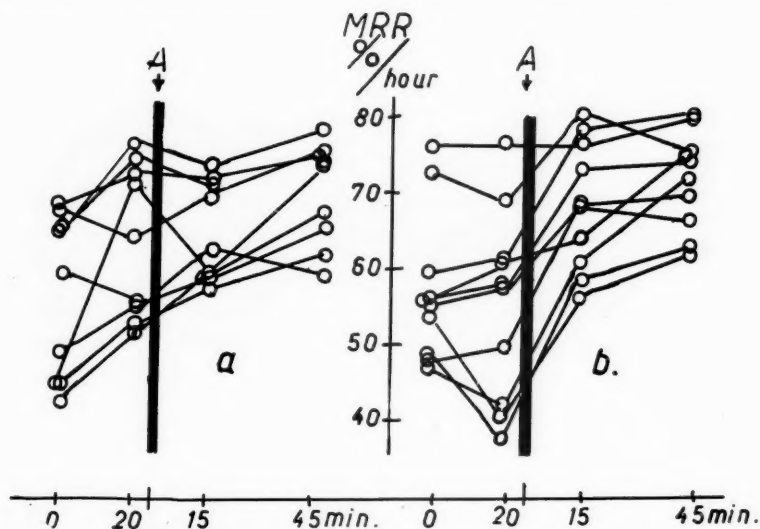
Fig. 1. — Methaemoglobin reduction rates (MRR) in red cells from restrained (a) and unstrained (b) rabbits before and after a subcutaneous injection of adrenaline (0.25 mg) at A.

In rabbit erythrocytes with glucose as substrate the enzymatic reduction of methaemoglobin is practically wholly inhibited by iodoacetic acid. Since the possibility of some reduction by other enzymatic or by non-enzymatic mechanisms e.g. by ascorbic acid, cannot be ruled out, their role in the increased rate of reduction, had to be considered. In 10 rabbits parallel determinations of MRR

in samples with an without iodoacetic acid were performed in
connection with the adrenaline experiments. The results are
summarized in table 2. In 8 out of ten samples taken before the
injection of adrenaline a small residual reduction of methaemoglobin

TABLE 2

(10 RABBITS)

| M R R in | M R R %/hour | | | t | p |
	Before	After Injection of Adrenaline	Increase		
1. Nitrite treated washed red cells + glucose + phosphate buffer pH 7.4 ..	57.9 ± 4.5	69.8 ± 3.0	11.9 ± 2.46	4.8	<0.001
2. Nitrite treated washed red cells + glucose + phosphate buffer pH 7.4 + 0.002 M iodo-acetic acid	6.5 ± 1.7	9.8 ± 1.1	3.3 ± 1.21	2.73	~ 0.02

may be noted. In the samples taken after the injection of adrenaline
the residual reduction amounts to over 6 per cent in 9 out of ten,
and to over 10 per cent in 8 out of ten samples. Thus a significant
increase in the methaemoglobin reduction occurs as well in a system
poisoned with iodoacetic acid as in the intact system. However,
the increase is less than that occurring in the absence of iodoacetic
acid. Consequently the probably non-enzymatic mechanism can
hardly be entirely responsible for the increase seen in the intact
system.

Adrenaline added in vitro to the suspension does not in any way
affect the rate of methaemoglobin reduction. (Table 3.)

TABLE 3

| Date | Rabbit | M R R %/hour | | |
| | | Control | Adrenaline in vitro | |
			$2\gamma/2$ ml	$20\gamma/2$ ml
18.6	A 859	61.5	63.0	61.5
18.6	Ri	65.5	63.5	66.0

DISCUSSION

The findings of Matthies, and collaborators (8) that repeted bleedings with ensuing reticulocytosis increase the MRR in erythrocytes was also confirmed during the course of the experiments. Unfortunately, it makes it impossible to control the duration of the increased MRR, since the repeted withdrawal of blood for analysis of MRR will by itself lead to an increased rate of reduction.

The authors mentioned above relate the increased rate of reduction to the active metabolism of reticulocytes, the increase in MRR being proportional to the percentage of reticulocytes. Since a common feature in all instances in which an increased MRR was observed, viz. hypoxia, mobilization of adrenaline or injections of it, is a discharge of reticulocytes into the circulation, it is necessary to consider the quantitative relations between reticulocytes and the MRR. A simple calculation shows that the metabolism of reticulocytes and a difference in their MRR cannot be alone responsible for the enhanced MRR. If we assume that the reticulocytes reduce during one hour all methaemoglobin contained in them to haemoglobin and take as the initial »normal» MRR 54 per cent/ per hour and 1.5 per cent as the normal percentage of reticulocytes in the rabbit, we can calculate that a MRR of 60 per cent/hour would require 15 per cent of reticulocytes and a MRR of 80 per cent already over 55 per cent reticulocytes. Thus the mean increase found in the adrenaline experiments would require some 40 per cent of reticulocytes and that encountered in the low pressure experiments 20 per cent, both being in those experimental conditions obviously too high, probably impossible, values. Therefore, if the reticulocytes are to be made responsible for the effect on MRR, it must be assumed that they interact with the metabolism of mature cells, as postulated by Jung & al (6). However, the experimental conditions in the study of Jung & al. differ from ours in an important point: they determine the MRR without adding any substrate and may therefore record an interaction between neighbouring cells through diffusible metabolites. This can hardly occur when there is plenty of substrate available, as in our experiments. Other possibilities must, therefore be taken into account. The changes in composition of blood plasma: hyperglycemia etc. might evidently be ruled out since we deal with washed cells. The amount of available substrate, however, definitely

influences the rate of methaemoglobin reduction. Thus if the cells are incubated without glucose the MRR is only a fraction of that recorded with glucose. It is however not likely that profound alterations in the intracellular composition of the red cells could possibly occur during the 15—20 minutes which suffice to enhance the MRR. Variations in the permeability of the cell membrane on the other hand could affect the amount of available substrate within the cell and possibly exert some influence on the rate of reduction too. The question must however be left open until more direct evidence is available.

However, in one point the presented results seem quite clear cut: Whatever the mechanism of the variations in the rate of methaemoglobin reduction might be, the experiments evidently show *how necessary it is even in biochemical work with isolated, washed cells, to pay attention to the condition of the animal when the blood sample is taken*, since variations (and inconsistences) may easily be introduced by unappropriate handling of the animals.

SUMMARY

1. The rate of methaemoglobin reduction (MRR) in nitrite treated washed red cells of rabbits were studied in various physiological conditions.

2. After exposure to low atmospheric pressure (360 mmHg, 6 hours a day, during 6—8 days) the MRR was found to be increased from a mean rate of 54 per cent/hour to 64 per cent/hour, the increase $10.1\% \pm 1.72$ being highly significant (P < 0.001).

3. Restraining of the animals led to a similar increase in the MRR.

4. After injections of 0.25 mg of adrenaline an elevated MRR was likewise found in animals treated with extreme caution and sitting free on the table. The increase amounted to 14.6 ± 3.00 and in an other series of samples to 16.7 ± 2.46 per cent/hour, both highly significant differences (P < 0.001).

5. In the restrained rabbits in which the MRR was already to begin with high, no further elevation was produced by the injection of adrenaline.

6. The possible mechanisms of the increased rates of methaemoglobin reduction are discussed.

7. The necessity of paying attention to the condition and handling of the animals from which blood samples for biochemical work are taken, is emphasized.

REFERENCES

1. HIRSJÄRVI, EVA: Ann. med. exper. Fenn. 1953:*31:*Suppl. 3.
2. HIRSJÄRVI, EVA: Acta physiol. Scand. 1955:*34:*338.
3. HUNTER, F. T.: The Quantitation of Mixtures of Haemoglobin Derivatives etc. Springfield, Ill. 1951.
4. JALAVISTO, EEVA: Acta Physiol. Scand. 1953:*29:*314.
5. JALAVISTO, EEVA: Ann. med. exp. Fenn. 1954:*32:*178.
6. JUNG, F., H. MATHIES, and K. ONNEN: Arch. exp. Path. Pharmakol. 1954:*222:*474.
7. KÜNZER, W., and D. SCHNEIDER: Acta haematol. 1953:*9:*346.
8. MATTHIES, H., F. JUNG, and K. ONNEN: Dtsch. Gesundheitswesen 1953:*8:*1562.
9. MATTHIES, H., F. JUNG, and R. SCHÄFER: Arch. exp. Path. Pharmakol. 1955:*225:*352.
10. PIHA, S.: Studies on Blood Regeneration During Progressive Reticulocytosis Due to Repeted Bleedings. pp. 99. — Ann. Acad. Sci. Fenn. Ser A. II. Fasc. 80. Helsinki 1956.

EFFECT OF HEPARIN ON SERUM LIPID AND LIPOPROTEIN CHANGES CAUSED BY BLEEDING ANAEMIA IN RAT

by

MATTI MIETTINEN

Acute bleeding anaemia in the rat causes a significant increase in total serum lipids, phospholipids and cholesterol. Total lipids increase relatively most and phospholipids increase relatively more than cholesterol. Cholesterol and phospholipids increase chiefly in the $alpha_2$ lipoprotein fraction, while these lipids are increased relatively less in the beta lipoprotein fraction. However the amounts of cholesterol and phospholipids in $alpha_1$ lipoprotein decreased slightly in most cases (4). In rabbit the increase of cholesterol and phospholipids caused by bleeding anaemia was seen in beta lipoprotein (3).

When rabbit serum is turbid due to lipaemia caused by bleeding anaemia, the formation of clearing factor is disturbed and intravenous heparin has only a slight or no clearing effect in lipaemia and is followed only by a slight and transient decrease in all $S_{1.21}$ lipoprotein fractions (8).

According to the investigations of the present author intravenous heparin decreases markedly the total serum lipids in haemorrhagic lipaemia in rabbit. The decrease in cholesterol and phospholipids was very small. The electrophoretic mobility of alpha and beta lipoproteins increased greatly. The cholesterol and phospholipids decreased slightly in beta lipoprotein and frequently increased a little in alpha lipoprotein (6).

Since the fat metabolism in the rabbit differs markedly from that in the rat and since mast cells are more numerous in the rat than in the rabbit (1) it seemed desirable to study the effect of heparin on serum lipid and lipoprotein changes caused by bleeding anaemia in the rat.

MATERIAL AND METHODS

From the tail of ten white rats weighing 200—300 gr, 4—7 ml of blood was let daily. Three to four days after the first bleeding, when the serum showed lipaemic turbidity, a sample was taken for lipid and lipoprotein analyses and 5 mg of heparin (Medica) was injected subcutaneously. Two hours after the heparin injection a new sample was taken from all the rats and one day later from six rats. To three of these rats 5 mg of heparin was injected again and a sample for analyses was taken two hours after the injection.

The total lipids, the total amounts of cholesterol and phospholipids, and the distribution of sudanophilic material and phospholipids in alpha$_1$, alpha$_2$ and beta lipoproteins separated by paper electrophoresis were determined in all the rats before and after heparin. However the distribution of cholesterol was determined in four rats only. In three cases the serum was inadequate for determination of the distribution of phospholipids in the lipoprotein fractions two hours after heparin injection; in these cases these determinations were made one day after the injection.

Total lipids were determined by the method of Swahn (9) cholesterol and phospholipids by the method presented by Nikkilä (7) and the distribution of cholesterol and phospholipids in lipoprotein fractions separated by paper electrophoresis were determined by the method described by Miettinen (2) which is a modification of Nikkilä's method (7). Sudanophilic material in the lipoprotein fraction was determined by the method of Swahn (9) and the lipoprotein fraction were separated in the manner described by Miettinen (5).

RESULTS

Heparin injection into ten rats with lipaemia caused by bleeding anaemia decreased markedly the total serum lipids in all cases during two hours after the injection (mean from 725 to 210 mg%).
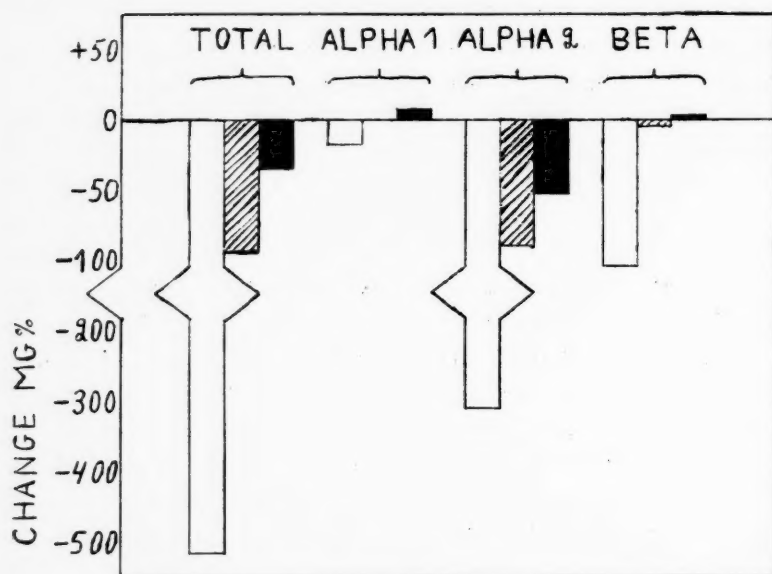
Fig. 1. — Changes in serum lipids and lipoproteins after heparin injection
into rats

☐ = total lipids (sudanophilic material);
/// = phospholipids; ■ = cholesterol.

The amounts of cholesterol and phospholipids decreased also, but
relatively less than the total lipids (mean of cholesterol from 117 to
83 mg % and of phospholipids from 240 to 150 mg %). Phospho-
lipids decreased relatively a little more than cholesterol. The turb-
idity of serum disappeared in most cases after heparin injection.

The cholesterol and phospholipids in the alpha$_2$ lipoprotein
fraction were decreased in all cases two hours after the injection
(mean of cholesterol from 103 to 50 mg % and of phospholipids
from 150 to 59 mg %). The changes in the cholesterol and phospho-
lipid amounts in the alpha$_1$ lipoprotein fraction (mean of cholesterol
from 28 to 36 mg % and of phospholipids from 50 to 50 mg %)
and the beta fraction (mean of cholesterol from 26 to 31 mg %
and of phospholipids from 43 to 37 mg %) were not significant.

The sudanophilic material in the alpha$_2$ lipoprotein fraction
decreased greatly in all cases (fig. 2). The sudanophilic material
in beta lipoprotein decreased also but relatively less than in alpha$_2$
lipoprotein. The sudanophilic material in alpha$_1$ lipoprotein fraction
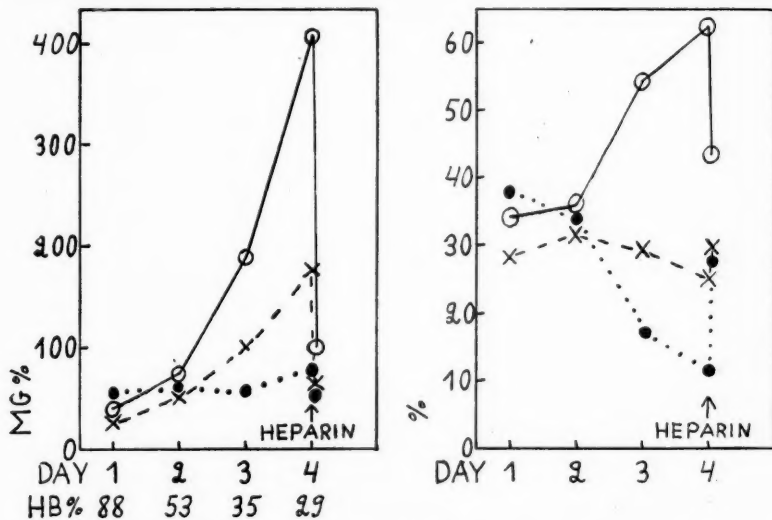
Fig. 2. — Changes in lipoproteins (sudanophilic material) in bleeding anaemia in 8 rats and the effect of heparin on these changes

●···●···● = alpha$_1$ lipoproteins: ⊙—⊙—⊙ = alpha$_2$ lipoproteins: ×——×——× = beta lipoproteins.

decreased in eight cases and increased in two cases. The relative amount of sudanophilic material increased in alpha$_1$ lipoprotein, decreased in alpha$_2$ lipoprotein, and was nearly unchanged in beta lipoprotein. One day after the injection the total lipids, cholesterol and phospholipids were in four cases higher and in two cases lower than two hours after the injection. However these lipids were in most cases markedly lower than before heparin injection.

The amount of phospholipids in the alpha$_1$ lipoprotein fraction was in all cases higher one day after the heparin injection than before the injection (mean 66 and 41 mg %). The amounts of phospholipids in alpha$_2$ and beta lipoproteins were in two cases higher and in three cases lower one day after the injection than before the injection. Heparin injection made one day after the first injection caused in two investigated cases a decrease of phospholipids in alpha$_1$ (mean from 68 to 35 mg %) and alpha$_2$ (mean from 90 to 48 mg %) lipoprotein and no change in the beta lipoprotein (mean from 32 to 30 mg %).

Heparin injection caused in no case a marked increase in the electrophoretic mobility of the lipoprotein fractions.

DISCUSSION

Heparin injection in lipaemia caused by bleeding anaemia in rats was followed by a marked decrease in total serum lipids. The amounts of cholesterol and phospholipids decreased relatively less than total lipids which indicates that heparin caused the most marked decrease in neutral fats. The decrease in total lipids was in most cases so great that after the heparin injection the sum of cholesterol and phospholipids was in many cases greater than the amount of total lipids.

Cholesterol and phospholipids decreased significantly only in the alpha$_2$ lipoprotein fraction. The sudanophilic material decreased greatly in the alpha$_2$ lipoprotein fraction, decreased markedly in the beta lipoprotein fraction and in most cases decreased slightly also in alpha$_1$ the lipoprotein fraction.

Since about 2—3 ml blood was needed for lipid and lipoprotein analyses, bleeding of such amount of blood may have caused at least partially the decrease in cholesterol and phospholipids. The great decrease in sudanophilic material in the alpha$_2$ lipoprotein fraction also caused a slight narrowing of this fraction which may play a part in the percentage decrease of this lipoprotein fraction.

In the rat the heparin injection did not cause a marked increase in the electrophoretic mobility of serum lipoproteins, as in the rabbit (6). In some rats the sudanophilic material decreased so much after the injection, that it was difficult to determine with certainty whether the electrophoretic mobility of lipoproteins had increased. However in many cases the margins of the lipoprotein fractions were detectable also after the injection and no significant increase was seen in the electrophoretic mobility of the fractions.

In normal rats the alpha$_1$ lipoprotein, which contains the major part of the serum cholesterol and phospholipids (5), migrates in electrophoresis with albumin or slightly faster than albumin. In the rabbit alpha$_1$ lipoprotein migrates with alpha$_1$ globulin. Heparin injection into rabbits with lipaemic serum increases the electrophoretic mobility of lipoproteins, so that alpha$_1$ lipoprotein migrates faster than albumin and a great part of beta the lipoprotein with the speed of alpha globulins (6). In rabbit the electrophoretic mobility of lipoproteins after heparin injection is more like that of rats.

There are also some other differences in the effect of heparin

injection on the changes in serum lipids and lipoproteins caused by bleeding anaemia in rat and rabbit. In th rat the total lipids decreased relatively more than in the rabbit. The changes in cholesterol and phospholipids in rabbit were small (6) but in rat the amount of cholesterol and phospholipids decreased in the alpha$_2$ lipoprotein fraction. In rats the number of heparin-building mast cells is greater than in rabbits (1). It is difficult to say if this has any effect on the above mentioned differences in the effects of heparin injection. In bleeding anaemia in the rat the neutral fats, cholesterol and phospholipids increase most in the alpha$_2$ lipoprotein fraction which has a lower molecular weight and a higher migration rate than the beta lipoprotein fraction. Possibly this plays a part in the greater decrease of these lipids after heparin injection in rat than in rabbit.

One day after the heparin injection the amounts of phospholipids in the alpha$_1$ lipoprotein fraction was higher than before heparin injection in all the investigated six cases. It is difficult to say if this increase is caused by the heparin for it seems possible that the cholesterol and phospholipid amounts in the alpha$_1$ lipoprotein fraction increase also without heparin injection on the fourth or fifth day after the beginning of the bleeding, after having been slightly decreased on third day (4).

SUMMARY

The effect of subcutaneous heparin injection on serum lipid and lipoprotein changes caused by bleeding anaemia was studied in ten rats. Two hours after the injection the total serum lipids were markedly decreased. Cholesterol and phospholipids decreased relatively less. The amounts of cholesterol and phospholipids decreased significantly only in the alpha$_2$ lipoprotein fraction while these lipids in the alpha$_1$ and beta lipoprotein fractions we were not changed significantly. Sudanophilic material decreased most in the alpha$_2$ lipoprotein fraction, decreased markedly in beta lipoprotein and in eight cases out of ten decreased in alpha$_1$ lipoprotein. The changes produced by heparin injection were opposite to those caused by bleeding anaemia.

REFERENCES

1. Constantinides, P.: Science 1953:*117*:505.
2. Miettinen, M.: Ann. med. int. Fenn. 1956:*45*:Suppl. 22.
3. Miettinen, M.: Ann. med. exp. biol. Fenn. 1957:*35*:Suppl. 10.
4. Miettinen, M.: Ann. med. exp. biol. Fenn. 1957:*35*:155.
5. Miettinen, M.: Ann. med. exp. biol. Fenn. 1957:*35*:168.
6. Miettinen, M.: Ann. med. exp, biol. Fenn. 1957:*35*:265.
7. Nikkilä, E.: Scand. J. Clin. Lab. Invest. 1953:*5*:Suppl. 8.
8. Spitzer, J. J., and Spitzer, J. A.: J. Lab. Clin. Med. 1955:*46*:461.
9. Swahn, B.: Scand. J. Clin. Lab. Invest. 1953:*5*:Suppl. 9.

EFFECT OF HEPARIN ON SERUM LIPIDS AND LIPO-PROTEINS IN HAEMORRHAGIC ANAEMIA

STUDIES ON RABBITS

by

MATTI MIETTINEN

In rabbits, haemorrhagic anaemia causes an increase in neutral fats, cholesterol and phospholipids in the serum (*e.q.* 1, 16). The increase in cholesterol and phospholipids occurs in the beta lipo-protein fraction, whereas the cholesterol and phospholipid amounts in the alpha lipoprotein decreased slightly in most cases (8). According to ultracentrifugal studies the amount of $S_{1.21}$ 73—300 lipoproteins increases in haemorrhagic anaemia in rabbits, while the other lipoprotein fractions do not show definite changes (15). Spitzer and Spitzer (14) reported that heparin does not clear serum which is lipaemic due to haemorrhagic anaemia, and that the ability to produce clearing factor declines as the serum becomes lipaemic. Before the onset of lipaemia and after its disappearance this capacity is normal. The authors suggested that lipaemia in haemorrhagic anaemia in rabbits may be ascribed to this circumstance.

MATERIAL AND METHODS

Blood was drawn from the ear vein of ten white rabbits, taking 25—60 ml daily. Haemoglobin determinations were made from these samples by the Sahli method. The results are stated in corrected Sahli values. In the first samples the haemoglobin

averaged 74 per cent and in the last samples before the
administration of heparin 24 per cent. When the rabbit serum
became turbid from lipaemia, 5 mg of heparin (Medica) was given
to the animals intravenously. Samples were then taken for lipid
and lipoprotein analyses one-half hour and in some cases also two
hours after the administration of heparin. From some of the rabbits
another sample was drawn on the following day, 5 mg of heparin
was again given intravenously, and one more bloodsample was
taken 2 hours later. The samples taken before and after heparin
administration were used for determination of the total serum lipids,
cholesterol and phospholipids and of the proportional distribution
of cholesterol and phospholipids in alpha and beta lipoprotein
fractions obtained with paper electrophoresis. The total lipids were
determined by the method of Swahn (17) and the total cholesterol
and phospholipids by the method described by Nikkilä (11).
Cholesterol and phospholipids in lipoprotein fractions were
determined by the method of Nikkilä (11) as modified by Mietti-
nen (7, 9).

<center>RESULTS</center>

The administration of heparin to rabbits with lipaemia due to
haemorrhagic anaemia caused a marked decline in the amount of
total lipids in all the rabbits within one-half hour after heparin
administration (mean from 824 to 528 mg per 100 ml). The
cholesterol declined very slightly in all cases (mean from 127 to
117 mg per 100 ml). The amount of phospholipids dropped slightly
in eight cases out of the ten (mean from 268 to 248 mg per 100 ml).
The changes in the cholesterol and phospholipid amounts were in
most cases within the limits of methodical error. The amounts of
cholesterol and phospholipids in the beta lipoprotein fraction
declined slightly in all cases (mean of phospholipids from 215
to 181 mg per 100 ml and of cholesterol from 113 to 100 mg per
100 ml). In the alpha lipoprotein fraction the cholesterol and
phospholipids increased slightly in most cases (mean of phospho-
lipids from 35 to 50 mg per 100 ml and of cholesterol from 11 to
15 mg per 100 ml).

In the rabbits whose serum before the administration of heparin
had been turbid from lipaemia the rate of migration of the alpha
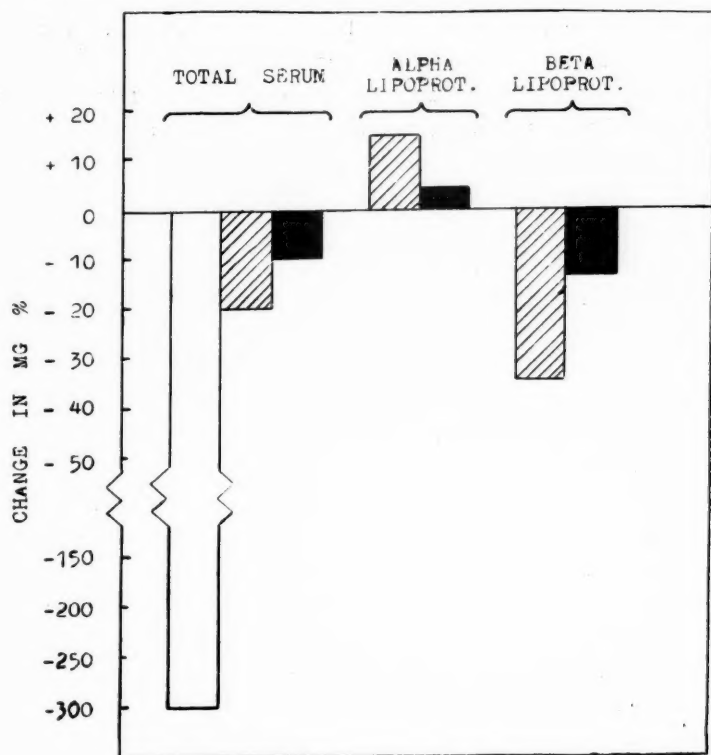
Fig. 1. — Changes in serum lipids and lipoproteins after heparin injection
in to ten rabbits

☐ total lipids (sudanophilic material)   ⦀ phospholipids
■ cholesterol

and beta lipoproteins was markedly increased after heparin. The
alpha lipoproteins migrated slightly rapidly than albumin, and the
boundary of the beta lipoprotein fraction on the anode side extended
into the alpha globulin zone. In those cases, on the other hand,
in which the serum had only been slightly opalescent from lipaemia,
the heparin did not cause such a change in the migration rate of
the lipoproteins.

The amount of total lipids was in most cases slightly lower two
hours after heparin administration than one-half hour after the
injection (mean from 573 to 512 mg per 100 ml). Twenty-four hours
after intravenous heparin the total lipids had again increased to the
pre-heparin level. A renewed injection of heparin once more reduced

considerably the amount of total lipids, as the first injection had done (mean from 1075 to 651 mg per 100 ml).

The corresponding changes in cholesterol and phospholipids were insignificant and were not as regularly seen as the changes in the total lipids.

DISCUSSION

The administration of heparin in lipaemia due to haemorrhagic anaemia in rabbits resulted in a marked decrease in the amount of total lipids, whereas only a very slight decrease occurred in the cholesterol and phospholipids. This indicates the possibility that heparin produced changes chiefly in the neutral fats.

In the clearing process the lipolysis of triglycerides results in an increased amount of free fatty acids (14). The injection of heparin in hyperlipaemia in man (4) and in the rabbit (2) increases the rate of migration of alpha and beta lipoproteins in electrophoresis. This is believed to be due to binding of the free fatty acids to alpha and beta lipoproteins (4). To this points also the fact that the addition of oleic acid to serum *in vitro* increases the rate of migration of lipoproteins in electrophoresis (6). Albumins bind free fatty acids and thus enhance the clearing process *in vitro* (3, 13). When the albumins no longer are capable of binding fatty acids which are liberated in the clearing process, the latter become bound to lipoproteins and the electrophoretic migration rate of the lipoproteins is increased (6).

According to Spitzer and Spitzer (15) the administration of heparin in haemorrhagic lipaemia in rabbits produces only a slight and transient decrease in all $S_{1.21}$ lipoprotein fractions. The poor ability of heparin to clear lipaemic turbidity in haemorrhagic anaemias they ascribed to an inability to produce clearing factor. In the rabbits in their series the amount of albumins had not declined during haemorrhagic anaemia, and therefore an albumin deficiency was not considered to be responsible for the poor clearing process. In the present investigation the turbidity of the serum did not in most cases decrease after heparin. When calcium soap formation was prevented by decalcification of serum, the density of the serum decreased in some cases after heparin injection.

According to Nikkilä and Gräsbeck (12) the turbidity of fasting nephrotic serum is only slightly cleared by heparin injection.

However, the formation of clearing factor was not disturbed in nephrosis. The authors suggested that the greatly reduced serum albumin becomes a limiting factor in the clearing process in nephrosis. Total lipids, cholesterol and phospholipids frequently decreased a little. Intravenous heparin increased the electrophoretic mobility of the beta lipoprotein component.

In the rabbit, trauma increases the serum phospholipids as well as S_f 0—12, S_f 12—20 and S_f 20—100 lipoproteins (10). The amount of phospholipids increased to four-fold and that of neutral fats to eight-fold within 48 hours after the trauma. Heparin injection partly inhibited this change and increased the rate of migration of alpha and beta lipoproteins in the electrophoretic field (5).

The observations referred to above and the findings in the present study seem to indicate that the administration of heparin in lipaemia due to haemorrhagic anaemia causes a marked decrease in the triglycerides because of their lipolysis. A part of the liberated fatty acids are probably boud to the lipoproteins, thus increasing the electrophoretic migration rate of the latter.

Cholesterol and phospholipids in the alpha lipoprotein fraction increased slightly in most cases in the present series, especially in cases in which evalution with the naked eye did not reveal an increase in the rate of migration of the lipoproteins. It is not possible to state whether the increase in the alpha lipoprotein amount was due to an actual conversion of beta lipoprotein to alpha lipoprotein. It may be possible that a part of the beta lipoprotein had migrated with the alpha$_1$ globulin in also those cases in which no increase in the migration rates of alpha and beta lipoproteins was discerned with the naked eye but in which a chemical analysis showed increased amounts of cholesterol and phospholipids in the alpha lipoprotein.

In the cases in which the alpha$_1$ lipoprotein migrated after heparin injection slightly more rapidly than albumins and in which the boundary of the beta lipoprotein fraction on the anode side extended into the alpha$_1$ globulin zone, the changes in the amounts of cholesterol and phospholipids in the alpha lipoprotein fraction were very small and whithin the limits of methodical error. About one-half of the cholesterol and phospholipids in the beta lipoprotein fraction had migrated with alpha$_1$ and alpha$_2$ globulins.

In those cases in which the rate of migration of the lipoprotein

increased after heparin injection, sudanophilic material accumulated over an about 3 mm wide zone on each side of the line of origin of the serum. Thus the amount of sudanophilic material absorbed into the paper at origin was practically not decreased when evaluated with the naked eye. The sudanophilic material therefore decreased chiefly in the beta globulin zone. Possibly the lipolytic action of the injected heparin was weaker against neutral fats absorbed into the paper at origin than against the smaller neutral fat particles in beta lipoprotein which had migrated with the beta globulin.

SUMMARY

The effect of intravenous heparin on changes in serum lipids and lipoproteins due to haemorrhagic anaemia was studied in ten rabbits. One-half hour after the heparin injection the amount of total lipids was significantly decreased. A slight decrease occurred in most cases in the cholesterol and phospholipid amounts. In those rabbits whose serum was turbid with lipaemia due to haemorrhagic anaemia the heparin injection considerably increased the rate of migration of the lipoproteins, so that the $alpha_1$ lipoprotein migrated slightly more rapidly than the albumin and the boundary of the beta lipoprotein on the anode side extended into the $alpha_1$ globulin zone. About one-half of the cholesterol and phospholipids in beta lipoprotein had migrated to the $alpha_1$ and $alpha_2$ globulin zones. The amounts of cholesterol and phospholipids in beta lipoprotein decreased slightly in all the cases. In the alpha lipoprotein fraction the cholesterol and phospholipid amounts increased slightly in most cases.

REFERENCES

1. BOGGS, T. R., and MORRIS, R. S.: J. Exp. Med. 1909:*11*:553.
2. COMFORT, A.: J. Physiol. 1955:*127*:225.
3. GORDON, R. S., Jr., BOYLE, E., BROWN, R. K., CHERKES, A., and ANFINSEN, C. B.: Proc. Soc. exp. Biol. & Med. 1953:*84*:168.
4. HERBST, F. S. M., LEWER, W. F., and LYONS, M. E.: J. Clin. Invest. 1955:*34*:581.
5. JOHNSSON, S. R., and WADSTRÖM, L. B.: Scand. J. Clin. Lab. Invest. 1956:*8*:323.
6. LAURELL, S.: Scand. J. Clin. Lab. Invest. 1955:*7*:28.

7. MIETTINEN, M.: Ann. med. int. Fenniae 1956:*45:*Suppl. 22.
8. MIETTINEN, M.: Ann. med. exp. biol. Fenniae 1957:*35:*Suppl. 10.
9. MIETTINEN, M.: Ann. med. exp. biol. Fenniae 1957. In press.
10. MILCH, L. J., REDMOND, R. F., and CALHOUN, W. W.: J. Lab. Clin. Med. 1954:*43:*603.
11. NIKKILÄ, E.: Scand. J. Clin. Lab. Invest. 1953:*6:*Suppl. 8.
12. NIKKILÄ, E., and GRÄSBECK, R.: Acta med. Scand. 1954:*150:*39.
13. ROBINSON, D. S., and FREUCH, J. E.: Quart. J. exper. Physiol. 1953:*38:*233.
14. SHORE, B., NICHOLS, A. V., and FREEMAN, L. W.: Proc. Soc. exp. Biol. & Med. 1953:*83:*216.
15. SPITZER, J. J., and SPITZER, J. A.: J. Lab. Clin. Med. 1955:*46:*461.
16. STARUP, U.: Undersøgelser over experimentel Hyperlipaemi. Munksgaard, København, 1927.
17. SWAHN, B.: Scand. J. Clin. Lab. Invest. 1953:*5:*Suppl. 9.

ÜBER DIE WIRKUNG DES PERORALEN ANTIDIABETICUMS N-[4-METHYL-BENZOLSULFONYL]-N'-BUTYLHARNSTOFF AUF SCHILDDRÜSENFUNKTION UND SERUMKOLESTERIN DER RATTE

von

VILJO ANTILA, A. N. KUUSISTO und G. HÄRTEL

Bei Untersuchung der Wirkung des oralen Antidiabeticums—N_1-sulfanilyl-N_2-n-butylcarbamid (BZ 55) — auf die Schilddrüsenfunktion der Ratte haben Kuusisto und Antila (4) eine strumogene Wirkung des Stoffes nachweisen können.

Härtel und Antila (3) haben festgestellt, dass BZ 55 bei Ratten Serumcholesterin und Gesamtlipoide zu erhöhen vermag.

Miller und Dulin (6) haben Eigenschaften des neuen oralen Antidiabeticums N-[4-methyl-benzol-sulfonyl]-N'-butylharnstoff untersucht und beobachteten dabei »a moderate enlargement of thyroid glands in all rats that were given the higher doses».

Da die beiden oral wirksamen antidiabetischen Stoffe N_1-sulfanilyl-N_2-n-butylcarbamid (BZ 55) und N-[4-methyl-benzol-sulfonyl]-N'-butylharnstoff (D 860) sich insofern voneinander unterscheiden, dass bei D 860 die para-ständige Aminogruppe durch eine Methylgruppe ersetzt ist, schien es von Interesse, auch die Wirkung von N-[4-methyl-benzol-sulfonyl]-N'-butylharnstoff (D 860) auf die Schilddrüsenfunktion der Ratte zu untersuchen

MATERIAL UND METHODEN

Für die Versuche wurden insgesamt 40 männliche Sprague-Dawley-Ratten verwendet, deren Gewicht 195—260 g betrug. Zehn Tiere erhielten Laboratoriumsfutter ad libitum. Zehn Tiere erhielten täglich 0,250 g Artosin[1] = D 860/kg Körpergewicht, welches einer kleinen Futtermenge beigemischt wurde. Erst wenn die Tiere diese Portion gefressen hatten, erhielten auch sie Laboratoriumsfutter ad libitum. Zehn weitere Tiere erhielten in gleicher Weise täglich 0,250 g D 860/kg Körpergewicht sowie, um optimale Resorption zu gewährleisten, ein Äquivalent $NaHCO_3$, und 10 Tiere erhielten täglich 0,5 g D 860/kg Körpergewicht sowie ebenfalls ein Äquivalent $NaHCO_3$. Die Tiere wurden in Abständen von 10 Tagen gewogen und nach 30 Tagen wurden sämtliche Tiere in Äthernarkose getötet, wobei Blut zur Bestimmung des Blutzuckers, des Serumcholesterins und des an Eiweiss gebundenen Jods (SPJ) entnommen wurde. Der Blutzucker wurde nach Somogyi (7) bestimmt, das Serumcholesterin nach der Methode von Sperry und Webb (8). Die Bestimmung des an Eiweiss gebundenen Jods erfolgte nach der Methode von Barker und Humbrey (1). Unmittelbar nach dem Töten wurden Schilddrüse, Thymus, Nebennieren und Hoden herauspräpariert, gewogen und in Bouins Lösung fixiert. Nach erfolgter Fixation wurden die Organe in Paraffin gebettet und geschnitten. Die Schnitte wurden mit Mallory Azan gefärbt und mikroskopisch untersucht. Für die Schilddrüsen wurde nach der histoquantitativen linearen Methode von Uotila und Kannas (9) das prozentuale Verhältnis von Epithel, Kolloid und Stroma ermittelt. Alle Ergebnisse vurden statistisch ausgewertet.

ERGEBNISSE

Während des Versuches nahm das mittlere Körpergewicht der Versuchstiere im gleichen Verhältnis zu wie das der Kontrollen. Die mittleren Gewichte von Nebennieren, Thymus und Hoden waren in allen Serien von gleicher Grössenordnung. Die mikroskopische Untersuchung ergab keine Veränderungen dieser Organe.

Die Blutzuckerwerte der Tiere mit D 860 lagen niedriger als

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[1] Artosin-N-[4-methyl-benzolsulfonyl]-N'-butylharnstoff-WZ der Firma C. F. Boehringer & Soehne, Mannheim — Waldhof.

die der Kontrolltiere. Für die Serie, die lediglich D 860 zugefüttert erhielt, betrug der mittlere Blutzucker $127,1 \pm 5,1$ mg%, für die Serie, die ausser D 860 ein Äquivalent NaHCO$_3$ bekam, $125,0 \pm 4,3$ mg%. Für die Serie mit 0,5 mg D 860/kg Körpergewicht und ein Äquivalent NaHCO$_3$ ergab sich ein Wert von $114 \pm 3,9$ mg%, wohingegen die Kontrolltiere einen mittleren Blutzucker von $146,3 \pm 4,3$ mg% aufwiesen. In sämtlichen Fällen war der beobachtete Unterschied zwischen Kontroll- und Versuchsserien signifikant ($p < 0,01$).

Die Serumcholesterinwerte der Versuchstiere waren im Vergleich zu den Kontrollen deutlich angestiegen. Kontrollserie: $39,2 \pm 1,04$ mg%, Versuchsserie mit 0,250 g D 860/kg Körpergewicht: $51,1 \pm 3,53$ mg%, Versuchsserie mit 0,250 g D 860/kg + NaHCO$_3$: $48,5 \pm 1,92$ mg% und Versuchsserie mit 0,5 g D 860/kg + NaHCO$_3$: $66,1 \pm 3,36$. Für den Unterschied zwischen Kontrollserie und Versuchsserie mit D 860 ist $p < 0,01$, für den Unterschied zwischen Kontrollserie und Versuchsserien mit D 860 und NaHCO$_3$ $p < 0,001$.

Hinsichtlich der Schilddrüsen lies sich weder makroskopisch noch mikroskopisch ein Unterschied zwischen den einzelnen Serien nachweisen. Die mittleren Tiergewichte, die absoluten und relativen mittleren Schilddrüsengewichte und die prozentuale Verteilung von Epithel, Kolloid und Stroma sind in der Tabelle 1 enthalten.

Hinsichtlich der relativen mittleren Schilddrüsengewichte ist zwischen Kontrollserie und Versuchsserien kein deutlicher Unterschied vorhanden. Auch was die prozentuale Verteilung von Epithel und Kolloid betrifft, lässt sich kein Unterschied nachweisen.

BESPRECHUNG

Nach Miller und Dulin (6) soll D 860 in grösseren Dosen (0,4 g/kg) bei Ratten eine mässige Vergrösserung der Schilddrüse verursachen. Bei der vorliegenden Untersuchung konnte auch für eine tägliche Dosis von 0,5 g D 860/kg Körpergewicht über 4 Wochen keine eindeutige Wirkung auf die Schilddrüse der Tiere nachgewiesen werden. Dagegen wurde in allen Serien, die D 860 erhielten, ein Ansteigen der Serumcholesterinwerte beobachtet.

Böhle und Mitarbeiter (2) haben festgestellt, dass die Serumcholesterinwerte von 20 Patienten während einer 4wöchigen Be-

TABELLE 1

Serien	Mittlere Tiergewichte zu Beginn des Versuches in g ± mittl. Fehler	Mittlere Tiergewichte am Ende des Versuches ± mittl. Fehler	Mittlere Gewichte der Schilddrüsen in mg ± mittl. Fehler	Mittlere Gewichte der Schilddrüsen in mg/100 g ± mittl. Fehler	Epithel in % ± mittl. Fehler	Kolloid in % ± mittl. Fehler	Stroma in % ± mittl. Fehler
D 860 0,250 g/kg n = 10	235 ± 3,7	256 ± 3,3	27,4 ± 1,5	10,71 ± 0,56	86,0 ± 0,84	5,3 ± 0,58	8,7 ± 0,39
D 860 0,250/kg + 1 Äq.NaHCO₃ n = 10	200 ± 1,6	252 ± 5,7	35,1 ± 2,2	14,03 ± 0,85	86,2 ± 0,85	3,8 ± 0,76	10,0 ± 0,75
D 860 0,5/kg + 1 Äq.NaHCO₃ n = 10	249 ± 4,5	267 ± 8,3	37,6 ± 2,0	14,72 ± 0,89	87,1 ± 0,92	4,7 ± 0,87	8,2 ± 0,57
Kontrollserie n = 10	223 ± 4,2	270 ± 12,3	30,7 ± 1,0	11,96 ± 1,58	85,8 ± 1,20	4,4 ± 0,91	9,8 ± 1,03

n = Anzahl der Tiere,

handlung mit D 860 abfielen. McGavack und Mitarbeiter (5) berichten jedoch, dass die Serumcholesterinwerte bei 7 von 25 behandelten Diabetikern anstiegen. Sie stellten weiterhin fest, dass D 860 die Aufnahme von Radiojod in der Schilddrüse verzögerte, wenn auch in geringerem Ausmass als BZ 55.

In ähnlicher Weise sprechen auch die vorliegenden Tierversuche dafür, dass D 860 wie auch BZ 55 eine serumcholesterin-erhöhende Wirkung besitzt und dass die strumogen-thyreostatische Wirkung von D 860 — falls eine solche vorhanden — jedenfalls schwächer ist als die von BZ 55.

ZUSAMMENFASSUNG

Das neue orale Antidiabeticum N-[4-methyl-benzolsulfonyl]-N'-butylharnstoff (D 860) verursachte bei Ratten, denen es 30 Tage lang in relativ hohen Dosen verabreicht wurde, eine deutliche Erhöhung des Serumcholesterins. Hinsichtlich Schilddrüsengewicht, des an Eiweiss gebundenen Serumjods und der prozentualen Verteilung von Epithel, Stroma und Kolloid der Schilddrüse konnten dabei zwischen Kontroll- und Versuchstieren keine Unterschiede nachgewiesen werden.

LITERATUR

1. BARKER, S. B., und HUMBREY, M. J.: J. Clin. Endocrin. 1950:10:1136.
2. BÖHLE, F., PFEIFFER, E. F., SCHÖFFLING, K., und STEIGERWALD, H.: Dtsch. med. Wschr. 1956:81:838.
3. HÄRTEL, G., und ANTILA, V.: Arzneim.-Forsch. 1956:6:701.
4. KUUSISTO, A. N., und ANTILA, V.: Acta Endocrinol. 1956:23:433.
5. McGAVACK, T. H., SEEGERS, W., HAAR, H., und ERK, V.: Metabolism 1956:5:919.
6. MILLER, L. W. Jr., und DULIN, W. E.: Science 1956:123:584.
7. SOMOGYI, M.: J. Biol. Chem. 1952:195:19.
8. SPERRY, W. M., und WEBB, M.: J. Biol. Chem. 1950:187:97.
9. UOTILA, U., und KANNAS, O.: Acta Endocrinol. 1952:11:42.

EFFECT OF ORAL ANTIDIABETIC AGENTS ON THE ACTIVITY OF THE THYROID

AN EXPERIMENTAL STUDY ON FROG TADPOLES

by

VILJO ANTILA

A recent report by Achelis and Hardebeck (1) has indicated that N_1-sulphanilyl-N_2-n-butylcarbamide (BZ 55) exerts an action on the rat thyroid, producing signs of hyperfunction in the histological picture such as are found also after administration of other sulphonamides.

In their studies of the effects of BZ 55, Kuusisto and Antila (3), using a quantitative histological method, observed that the drug appeared to have an action similar to that of goitrogenic substances.

Experimenting on rats, Miller and Dulin (4) found that N_1-p-tolylsulphonyl-N_2-n-butylcarbamide (D 860), administered in large doses, caused a moderate enlargement of the rat thyroid.

Using a quantitative histological method, Antila, Kuusisto and Härtel (2), however, found no evidence that the action of D 860 on the rat thyroid was similar to that of goitrogenic substances.

In view of the close relationship that exists between the metamorphosis of the frog tadpole and the activity of its thyroid gland, the present study was undertaken in an attempt to find out whether BZ 55 and D 860 have a retarding effect on the metamorphosis of the tadpole.

MATERIAL AND METHODS

The experiment was carried out on tadpoles of *Rana temporaria*. The tadpoles were collected at one time and place, and all of them were kept under the same external conditions in shallow glass jars containing ten litres of tap water. They were fed a constant diet of dry powdered liver daily. The water in the jars was changed every three days.

For the experiment 150 tadpoles of equal size were selected and divided into three groups of 50, which were placed in the glass containers. BZ 55, dissolved in 1-n NaOH solution, was added into one container in the ratio of 1 : 100,000; D 860 similarly dissolved was added into the second container in the same ratio; and a corresponding amount of the vehicle alone was added into the third container. The tadpoles were kept in these solutions for three days, and then the water was changed.

The development of the tadpoles was closely observed. On the appearance of the forelegs the tadpoles were killed in a small amount of water containing a few drops of chloroform. Immediately thereafter they were measured (from head to tail), dried on blotting-paper, and weighed, and then fixed in Bouin's fluid. After fixation they were suspended in paraffin. Histological sections were stained by the hematoxylin-eosin method. The preparations were studied under the microscope, and the colloid content of the thyroids was measured with a planimetre.

Student's *t* test was applied to all statistical data.

RESULTS

The metamorphosis of the frog tadpoles that were kept in solutions of BZ 55 and D 860 began later than that of the controls. On the appearance of the forelegs the treated tadpoles were also heavier and longer than the tadpoles of the control group.

Table 1 shows the time from the beginning of the experiment to the appearance of the forelegs.

The mean weights and lengths of the tadpoles on the appearance of the forelegs are given in Table 2.

The difference in the mean weight between the tadpoles treated with BZ 55 and the controls is statistically significant, and the

TABLE 1

APPEARANCE OF THE FORELEGS

	Days from the beginning of the experiment					
	Under 20	20	25	30	35	40
BZ 55 group ..		1	8	26	13	2
D 860 group			4	20	21	5
Control group ..	14	24	10	2		

TABLE 2

MEAN WEIGHTS AND LENGTHS OF TADPOLES TREATED WITH BZ 55 AND D 860

	Control group	BZ 55 group	D 860 group
Weight, mg	437 $\pm$ 15.5	485 $\pm$ 11.5	570 $\pm$ 20.0
Length, mm	36.9 $\pm$ 0.75	39.0 $\pm$ 0.81	41.4 $\pm$ 0.85

difference in the mean weight between the group treated with D 860 and the control group is statistically highly significant.

The differences in the mean length between the three groups are statistically highly significant.

Microscopic examination of the thyroids revealed no difference between the three groups in the colloid content of the thyroids as estimated by the planimetric method.

DISCUSSION

The experiment shows that both BZ 55 and D 860, acting in the same way as do goitrogenic substances, have a retarding effect on the metamorphosis of the frog tadpole.

Previous studies of the effects of BZ 55 have demonstrated that this drug has a moderate goitrogenic action, whereas experiments with D 860 have failed to prove beyond doubt that D 860 exerts such an action. The present investigation, however, supports the view that D 860 also exerts a moderate goitrogenic action on the thyroid.

SUMMARY

The action of BZ 55 and D 860 on the metamorphosis of the frog tadpole was studied in a group of 150 tadpoles. Both substances

were found to retard the metamorphosis of the tadpole, their action being thus similar to that of goitrogenic substances. On the appearance of the forelegs the tadpoles treated with BZ 55 and D 860 were heavier and longer than the controls. Microscopic examination of the thyroids showed no difference between the treated tadpoles and the controls.

REFERENCES

1. ACHELIS, J. D., and HARDEBECK, K.: Deutsche med. Wchnschr. 1955: *80*:1452.
2. ANTILA, V., KUUSISTO, A. N., and HÄRTEL, G.: To be published.
3. KUUSISTO, A. N., and ANTILA, V.: Acta Endocrinol. 1956:*23*:433.
4. MILLER, W. L., JR, and DULIN, W. E.: Science 1956:*123*:583.

OBSERVATIONS ON SKELETAL CHANGES IN EXPERIMENTAL LATHYRISM IN THE RAT

by

OLAVI SARPIO

In recent years much attention has been paid to the treatment of scoliosis of the spine. In spite of the great progress made, there are many questions still to be answered. It appears, therefore, that continued clinical and experimental research is needed to develop the methods of treatment of this severe disease, which often causes serious invalidity so that enduring results may be achieved.

The aim of the experiment reported in the following was to ascertain the nature of artificially produced scoliosis of the spine and to lay a foundation for further study of the methods of treatment in experimental animals.

For several decades now, epidemic lathyrism has been known to exist in some parts of India and in some Mediterranean countries. It appeared especially during times of famine, attacking the poorer elements of the population particularly. Grain shortage forced the people to include in their diet some legumes. These were observed to cause severe poisoning in humans and in domestic animals. These legumes, which under normal conditions were part of the normal diet of those with a lower standard of living, are mainly of the Lathyrus-species. For this reason the disease was later known as Lathyrism, also.

Stockman (10) was the first to present any data on this disease when he described its appearance in India. The first symptoms

of the disease were usually muscular weakness and, in the later stages, spastic paralysis in the lower extremities. In more severe cases, urinary and fecal incontinency often occurred. Geiger, Steenbock and Parsons (2) noted scoliosis and hernia in white rats fed on a diet containing 50 per cent sweet pea (Lathyrus odoratus) seed. They also found that the toxic factor was soluble in water. Lewis *et al.* (4) noted that some other Lathyrus-species (Lathyrus hirsutus, L. tingitans, L. sphaericus and L. sylvestris Wagneri) also contained a factor that caused similar changes in rats. In Spain, Vivanco and Diaz (11) have published studies on local epidemic diseases caused by legumes. Lee (3) reported lathyric syndromes in experimental animals fed an a diet of 50 per cent singletary pea (Lathyrus pusillus) seed. Together with Dupuy (1). he extracted the toxin in crystalline form from the singletary pea. In the same year, Schilling extracted crystals from sweet pea which caused skeletal changes in rats. Schilling and Strong (8) finally proved that β-aminopropionnitril was the toxic factor causing lathyrism. This chemically pure substance caused the same changes in experimental animals as did the finely-ground sweet pea meal used in earlier experiments.

Ponseti *et al* (6) have especially investigated the skeletal changes in experimental animals caused by Lathyrus odoratus seeds. They observed that a diet consisting of 50 per cent Lathyrus odoratus seeds caused kyphoscoliosis, severe periosteal new-bone formation and, in some animals aneurysm of the aorta. In an investigation published in 1954, they dealt with the histopathology of the skeletal lesions and observed several similarities in some bone diseases in humans and in the skeletal lesions caused by lathyrism in experimental animals.

PRESENT OBSERVATIONS

The aim of the following investigation is to study further the skeletal changes in hip and spine caused by lathyrism.

Tests were performed on 30-day-old white male rats of the Spraque-Dawley strain each of them weighing c. 38.5 g when the experiment was commenced. Experimental group, consisting of 20 animals, was fed on a diet containing Lathyrus odoratus seed. The experimental diet was as follows:

Lathyrus odoratus meal 50%
Casein 10%
Sucrose 24%
Brewer's yeast 10%
Salt mixture (Mc Collum)...................... 4%
Vitamin mixture (Olive oil containing 0.21 mg vita-
   min A, 0.26 IU vitamin D, 10 mg vitamin E and
   0.15 mg vitamin K per kilogram of diet).... 2%

In the control group (10 rats) commercial pea meal (Pisum sativum) was substituted for the Lathyrus odoratus meal. All the test animals were allowed to eat and drink *ad libitum*. The animals were weighed every ten days. During the experiment seven rats died spontaneously and the other were sacrificed at intervals of five days. Thus the last animal to remain alive was given Lathyrus odoratus diet for 60 days. Both hip joints with their acetabula and also the proximal part of the femur were removed. At the same time specimens of the spine were preserved, with special attention being paid to the thoracic spine, where most of the macro-scopically observed changes occurred. The specimens were fixed in 10 per cent formaline; haematoxylin-eosin was used for staining.

Clinically, the animals fed a Lathyrus-diet already showed signs of illness within one to two weeks. Later on, they became sluggish and ungainly. Compared with the controls their average weight was obviously lower (Table 1). In three to four weeks, macroscopic scoliosis was observed. Later four rats developed paraplegia in the hind quarters and urinary and fecal incontinence.

At necropsy noticeable skeletal lesions were observed in rats fed on sweet pea seed diet for 2—3 weeks. At an earlier stage kyphoscoliosis developed at the thoracolumbar junction (Fig. 1), but after a longer period of diet, changes were usually observed in the mid-thoracic spine. These were almost always followed by vertebral rotation. The rotation of the vertebrae was accompanied by secondary sternal and costal deformity. Simultaneously wide periosteal new-bone formations were observed, particularly at the sites of the ligamentous and tendinous insertions in the perios-teum of the bones. Three rats died spontaneously four to five weeks after being put on diet of Lathyrus odoratus seed. Death was due

TABLE 1
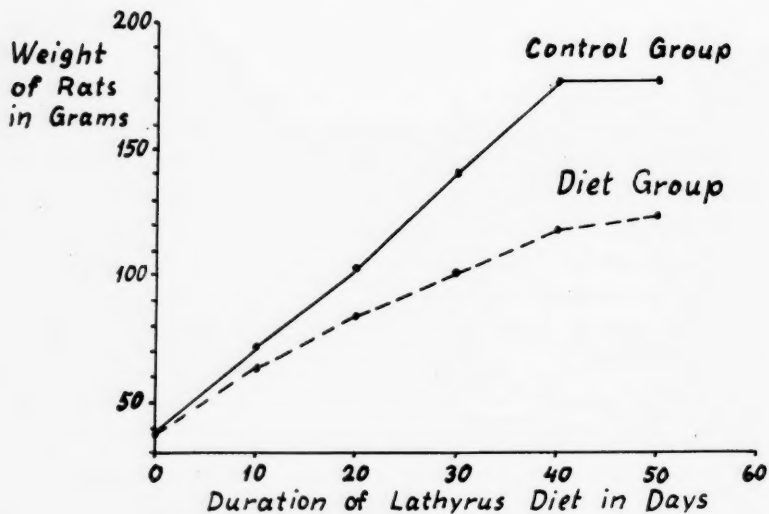
Fig. 1. — Roentgenogram of the skeleton of an 80-day-old test animal. Scoliotic changes at the thoracolumbar junction are apparent. Secondary deformity of the rib bones. The rat was fed on diet for 50 days.
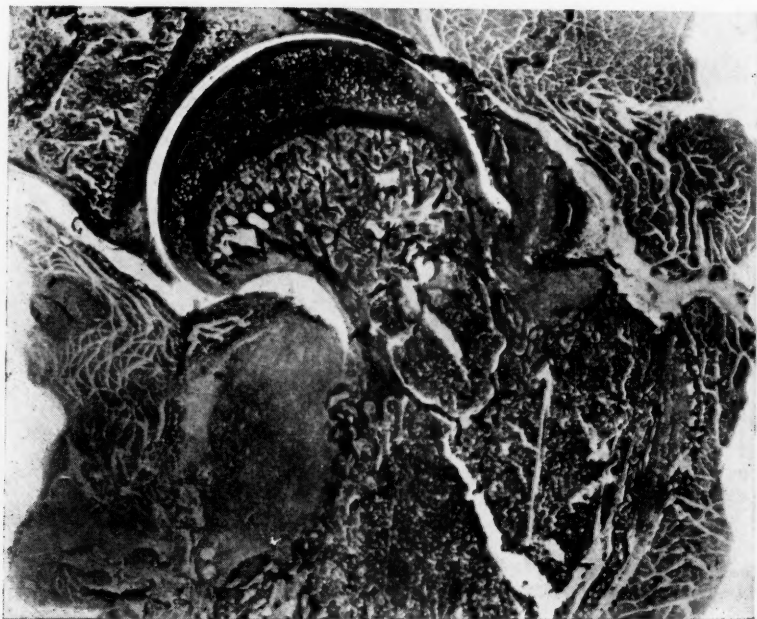
Fig. 2. — Upper portion of the femur and the hip joint of a 56-day-old rat which had been fed on Lathyrus-diet since the age of one month. In the picture strong subperiosteal new-bone formations with detachment of periosteum are clearly apparent. × 10.

Fig. 3. — Sagittal section of the vertebrae of a rat fed on diet for 26 days. In the centre of the picture a slipped and collapsed vertebra. × 10.
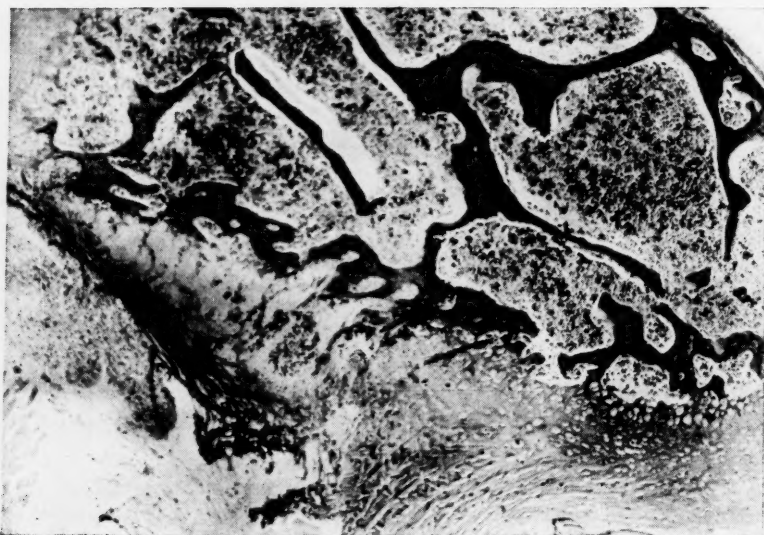
Fig. 4. — Enlargement of a part of Fig. 3 (× 45). Distortion of epiphysis and detachment of the periosteum of ventral surface of the vertebra can be seen.

to aortic aneurysm. At necropsy no clear cause of death was apparent in the four remaining rats.

Already within 2—3 weeks, histopathological examination of the hip joints, proximal femur and spine of all the experimental animals revealed periosteal new-bone formation. At the commencement of the experiment very cellular connective tissue developed at the sites of the tendinous insertions underneath the periosteum (Fig. 2). Later on, these were seen to form cartilage and trabeculae developed adjacent to the bone. After the rats were put on diet for more than four weeks the new-bone formation became rather pronounced in some of them. In none of the animals in this group did slipping of the femoral upper epiphysis take place.

After three weeks of diet skeletal lesions of the vertebrae were observed. There was collapse of the anterior portion of the vertebral bodies near the site of epiphyseal plates with resulting kyphosis (Fig. 3). Also after four weeks of diet, scoliosis developed. At this stage of the deformity some detachment of the periosteum at the sites of tendinous insertions of intervertebral ligaments was observed (Fig. 4). In each rat only of few of these wedge-shaped

Fig. 5. — Sagittal section of thoracic vertebrae of a 75-day-old rat fed on Lathyrus-diet for 45 days. Vertebrae distorted. Medulla at the site of kyphosis compromised. × 10.

Fig. 6. — Enlargement of a part of Fig. 5 (× 45). Epiphyseal distortion and beginning of epiphyseolysis.

vertebrae were usually observed in the largest kyphoscoliotic deformation area. Only in three rats did clear slipping of the epiphysis of the vertebral body occur (Fig. 5, 6). The intervertebral discs did not appear to have undergone any pathological changes.

After three weeks of diet, deformities of the thorax and the sternum could be observed in all the test animals.

DISCUSSION

Severe skeletal lesions are often reported to be the main symptoms of experimental lathyrism in rats fed on Lathyrus-diet for some weeks (2, 4, 5, 6, 11). In the present investigation pronounced periosteal new-bone formation and kyphoscoliotic deformation of the spine were also observed in test animals. Osteoporosis of the bones does not necessarily result from lathyrism but it may be a secondary phenomenon caused by the inactivity of the animals.

It seems that the new-bone formation in the long bones, such as the femur, begins at the site of the tendinous insertions underneath the periosteum. It, therefore, appears that the detachment of the periosteum is a decisive factor in the deformity. Metaphyseal detachment of the periosteum and proliferating connective-tissue formation underneath the periosteum could also be seen. Later on, new-bone formation could be observed in the connective-tissue. It is possible that the severe kyphoscoliosis may be due to the collapse of the vertebrae together with the loosening of the intervertebral ligamentous insertions causing the severe rotatory component of the deformity.

The lack of epiphyseolysis at the upper end of the femur is similar to that reported by Ponseti (5), who noted only a few cases of slipping of the femoral epiphysis in a group of test animals fed a diet since the age of three weeks. In the present investigation on a group of animals put on diet at the age of one month, slipping of the vertebral epiphysis occurred only a few times. Ponseti also noted only a few cases — and they were mild — of epiphyseolysis in animals of the same age.

It is interesting to speculate on the possible relationship between the skeletal lesions caused by experimental lathyrism in test animals and some diseases of unknown etiology and pathogenesis in humans. Ponseti (5) suggests that the so-called idiopathic scoliosis in humans is caused by the weakening of the epiphyseal plates and

by the simultaneous loosening of ligamentous insertions of the vertebrae. The slipping of the upper femoral epiphysis which occurs in adolescents is a similar phenomenon to the epiphyseal changes produced by lathyrism in experimental animals (7). In this work they also observed disturbances in the protein metabolism of the patients. Similar changes have been also observed in idiopathic scoliosis-patients with an abnormal secretion of some aminoacids into the urine (9).

The skeletal lesions in experimental rats and the aforementioned similar lesions in humans need not suggest the same pathogenesis. There is, however, need for further study of the metabolism of these diseases. It is possible that the underlying cause of the disease may be some factor in the diet that disturbs the normal protein metabolism.

SUMMARY

Diets containing 50 per cent Lathyrus odoratus seeds fed to rats at the age of one month produced wide subperiosteal new-bone formations in the long bones, especially at the sites of tendinous insertions. Severe kyphoscoliotic deformities of the spine resulted from the weakening and collapse of several vertebrae. It is surmised that scoliosis produced in experimental animals is due to the loosening of the insertions of the intervertebral ligaments of the vertebrae.

REFERENCES

1. Dupuy, H. P., and Lee, J. G.: J. Am. Pharm. Assoc. Sci. Ed 1954: 43:61.
2. Geiger, B. J., Steenbock, H., and Parsons, H. T.: J. Nutrition. 1933:6:427.
3. Lee, J. G.: J. Nutrition. 1950:40:587.
4. Lewis, H. B., Fajans, R. S., Esterer, M. B., Shen, C. W., and Oliphant, M.: J. Nutrition. 1948:36:537.
5. Ponseti, I. V., and Shepard, R. S.: J. Bone and Joint Surg. 1954: 36—A: 1931.
6. Ponseti, I. V., and Baird, W. A.: Am. J. Pathol. 1952:28:1059.
7. Ponseti, I. V., and Mc Clintock, R.: J. Bone and Joint Surg. 1956: 38—A:71.
8. Schilling, E. D., and Strong, F. M.: J. Am. Chem. Soc. 1954:76:2848.
9. Stearns, G., Jo-You Tung Chen, Mc Kinley, J. B., and Ponseti, I. V.: J. Bone and Joint Surg. 1955:37—A:1028.
10. Stockman, R.: J. Pharm. Exp. Ther. 1929:37:43.
11. Vivanco, F., and Diaz, C. J.: Bull. Inst. Med. Res., Madrid. 1951:4:1.

EFFECT OF SOMATOTROPIN (STH) ON THE MATERNAL ORGANISM DURING PREGNANCY

by

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It has been suggested that the somatotropic hormone excreted excessively from the maternal anterior pituitary in prediabetic and diabetic states may be responsible for the increased weight of fetuses.

Experimental support for this hypothesis was offered by, *e.g.*, Teel (21), Hain (9), Sontag *et al.* (20) and Barns *et al.* (1) by means of administration of growth hormone-containing anterior pituitary extracts to pregnant rats. It is to be observed, however, that frequently in these experiments the duration of the pregnancy was longer and the number of fetuses was smaller in the group which was administered hormones. In the studies of Engfeldt and Hultquist (11, 4), in which highly purified STH preparations were used, the number of fetuses and the duration of pregnancy were similar in the STH treated and control groups. The mean weight of fetuses, however, was elevated in the STH treated group.

The mode of action of STH on the growth of fetuses is still unknown. It has been stated that the anterior pituitary hormones are not able to cross the placenta. This has been shown to be the case with ACTH (17, 22, 23, 18, 3, 12) and the thyrotropic hormone (16). The effect of these hormones and thus probably also that of STH (13) on fetuses could therefore only be secondary to the changes induced by these hormones in the maternal organism.

The normal rat reacts to the typical weight-increasing effect of STH on body and visceral weights (5). An intact rat, however,

is known to be highly resistant to the diabetogenic effect of STH. This effect of STH in the rat is observable only when large amounts of the hormone are administered to partially depancreatized or alloxan-diabetic rats (14, 2, 8, 15).

In view of the above it was considered to be of interest to study whether the rat organism is more sensitive to STH during pregnancy.

MATERIAL AND METHODS

A total of 102 female rats of Wistar strain of the same age (weighing 120—180 gm) were used in this experiment. To 25 pregnant and 26 non-pregnant rats ware given 30 tibia units of STH (Somacton, Nordiska Hormonlaboratoriet, Malmö, Sweden) in a prolonged action solvent intramuscularly once a day. A group of 26 pregnant and 25 non-pregnant rats that received no STH served as controls.

The rats were weighed weekly during pregnancy. They were killed 6 hours after delivery and the liver, spleen and the fetuses were weighed.

The fasting blood sugar level was determined by the method of Somogyi-Shaffer-Hartman (19) weekly during pregnancy and after delivery.

RESULTS AND DISCUSSION

Table 1 shows the mean body weights in the different groups at the beginning of the experiment and 6 hours after delivery.

TABLE 1

	Pregnant		Non-Pregnant	
	STH	Control	STH	Control
Body weight				
At the beginning of experiment, gm	147	147	154	147
At the end of experiment, gm	168	161	168	164
Mean weight increase, gm	19.8	16.2	14.1	17.9
Number of animals	25	26	26	25
Standard deviation	20.2	9.2	12.1	13.4
Standard error	2.79	2.53	2.41	2.73

No statistical difference is observed on an average (P < 0.1) between the different groups on comparison of the mean increases in the body weight of the animals.

The duration of pregnancy and the number of fetuses was the same in both groups. In the STH-treated group the duration of pregnancy was 21.2 days and the number of fetuses 188; in the control group these were 20.8 days and 189 fetuses.

The fasting blood sugar values also showed no statistically significant difference.

The weights of the liver and spleen in the different groups and the mean average weight of the fetuses are given in table 2.

TABLE 2

	Pregnant		Non-Pregnant	
	STH	Control	Somacton	Control
Liver				
Number of animals	25	26	26	25
Mean weight, gm	8.28	7.45	6.88	7.07
Standard deviation	1.43	1.14	0.88	1.10
Standard error	0.28	0.22	0.17	0.22
Spleen				
Number of animals	24	26	26	24
Mean weight, gm	0.88	0.74	0.84	0.81
Standard deviation	0.23	0.20	0.16	0.17
Standard error	0.047	0.040	0.033	0.036
Mean weight of fetuses				
Number of litters	25	25		
Mean of litter averages ..	5.28	4.87		
Standard deviation	0.51	0.56		
Standard error	0.10	0.11		

STATISTICAL ANALYSIS

In comparing the mean gland weights of the different animal groups, the statistical technique known as variance analysis was applied (6). In the first stage, the amount of variation between pregnant and non-pregnant rats was determined, then, similarly, the amount of variation within these main groups between the STH and control groups was determined. In testing these variances

against the residual variance, using the appropriate v^2 or variance ratio test, the following results were obtained:

Liver Weight

Difference between pregnant and non-pregnant rats was highly significant ($P < 0.0005$).

Difference between STH and control group was almost significant in general ($P < 0.05$); however, it was significant ($P < 0.025$) in pregnant rats and non-significant ($P < 0.1$) in non-pregnant rats. Thus a certain interaction between treatment and pregnancy is observable.

Spleen Weight

There was no statistically essential difference between pregnant and non-pregnant rats ($P < 0.1$).

The difference between STH and control groups was non-significant on an average ($P \gtrless 0.1$); however it was significant in pregnant rats ($P < 0.025$) and non-significant ($P < 0.1$) in non-pregnant rats. A definite interaction cannot be established.

In testing the effect of STH treatment on the mean weight of the offspring, »Student's t-test» (7) was applied, and the difference of means appeared to be significant on the level ($P < 0.01$).

Thus it is observed that the dosage of STH used, which was unable to effect any change in weights of the liver and spleen of non-pregnant rats, caused a significant difference in the liver weight of pregnant STH-treated rats as compared to all the controls, and in the spleen weight as compared to pregnant controls. The results suggest that at least the effect of STH on organ weight is potentiated by pregnancy. The fasting blood sugar level and body weight were not influenced by a similar STH treatment either in pregnant or in non-pregnant rats.

SUMMARY

A highly purified STH preparation was given daily during three weeks to pregnant and non-pregnant rats. The following effects were observed:

The fetal weight was increased by STH treatment without changes in the duration of pregnancy or in the number of fetuses.

A significant increase in the weight of the liver and the spleen was observed in the STH treated pregnant rats as compared to pregnant controls.

It is suggested that the rat organism is rendered in some respects more sensitive to STH by pregnancy.

For the Somacton, STH preparation, we are indebted to Dr. F. Paulsen, Nordiska Hormonlaboratoriet, Malmö.

REFERENCES

1. BARNS, H. H. F., and SWYER, G. I. M.: Brit. Med. J. 1952:4790:914.
2. BENNETT, L. L., and LI, C. H.: Am. J. Physiol. 1947:150:400.
3. DOMM, L. V., and LEROY, P.: Anat. Record. 1951:109:395.
4. ENGFELDT, B., and HULTQVIST, G. T.: Acta endocrinol. 1953:14:181.
5. EVANS, H. M., SIMPSON, M. E., and LI, C. H.: Growth 1948:12:15.
6. FISHER, R. A.: Statistical Methods for Research Workers, Oliver & Boyd, London & Edinburgh, 1950, P. 222 et seq.
7. FISHER, R. A.: Ibidem, P. 119 et seq.
8. GAARENSTROOM, J. H., HUBLE, J., and DE JONGH, S. E.: J. Endocrinol. 1949:6:71.
9. HAIN, A. M.: Quart. J. Exp. Physiol. 1932:22:71.
10. HULTQVIST, G. T.: Acta pathol. microbiol. Scandinav. 1948:25:131.
11. HULTQVIST, G. T., and ENGFELDT, B.: Acta endocrinol. 1949:3:365.
12. JOST, A.: Biol. Med. (Paris) 1951:40:205.
13. JOST, A.: Rec. Progress Hormone Res. 1953:8:379.
14. MARX, W., ANDERSON, E., FONG, C. T. O., and EVANS, H. M.: Proc. Soc. Exp. Biol. & Med. 1943:53:38.
15. MILLMAN, A. E., and RUSSELL, J. A.: Endocrinology 1950:47:114.
16. PETERSON, R. R., and YOUNG, W. C.: Endocrinology 1952:50:218.
17. RAYNAUD, A.: Bull. soc. zool. France 1943:68:131.
18. RAYNAUD, A., and FRILLEY, M.: Compt. rend. 1950:230:331.
19. SOMOGYI, M.: J. Biol. Chem. 1930:86:655.
20. SONTAG, L. W., and MUNDSON, P. L.: Am. J. Physiol. 1934:108:593.
21. TEEL, H. M.: Am. J. Physiol. 1926:79:170.
22. WELLS, L. J.: Anat. Record. 1947:97:409.
23. WELLS, L. J.: Proc. Soc. Exp. Biol. & Med. 1948:68:487.

GLUCOSE AND STARCH TOLERANCE TEST

IN EXPERIMENTAL PANCREATITIS PRODUCED WITH ETHIONINE

by

A. A. HALME, O. J. KASSILA, and K. J. W. HARTIALA

Some years ago an important new tool for the experimental study of pancreatic function and dysfunction has come into use in the form of the synthetic amino acid, ethionine (alpha-amino-gamma-ethylmercaptobutyric acid). It has been shown that feeding or injection of ethionine produces pancreatic lesions in animals, and its specificity for the acinar cells of the pancreas has been described in detail (1, 3, 6, 7, 8, 9, 11, 13, 15, 16, 17, 19, 20). The lesions vary from atrophy of the gland to severe pancreatitis with fat and hemorrhagic necrosis, depending upon the dosage schedule of administration. The ducts, blood vessels and islets have shown no significant histological changes.

Injection of a single large dose of ethionine into dogs produces impairment of pancreatic enzyme secretion without suppression of volume flow and without histologic damage. This effect is reversible by simultaneous injection of an equimolar amount of methionine (14). Contrasting to this selective depression on enzyme secretion, the animals which have received ethionine for a number of days show a tendency for depression of all components of the pancreatic juice. However, the volume and bicarbonate output is less impaired than the secretion of organic material (1, 11). When doses of ethionine which will eventually produce severe necrosis of the gland are given daily, there is a fall in serum amylase during

the first few days, followed by a transient elevation and return to
subnormal levels. The transient elevation corresponds to the period
when maximal rate of disintegration of acinar cells is seen histo-
logically (11, 13).

The principal factors which have been recognized as possible
etiologic agents in pancreatitis are obstruction, regurgitation of
bile, vascular occlusion and trauma. The new evidence indicates
that in addition to these more or less mechanical factors a metabolic
disorder may also contribute. Ethionine is a powerful tool with
which this newly recognized factor can be explored experimentally.

Recently, Althausen and Uyeyama (2) have reported their
experience in the patients with chronic pancreatic disease. They
have used a test which they call »the starch tolerance test» and
»a new test of pancreatic function based on the amylolytic activity
of pancreatic juice in the intestine». They feed a lemon-extract-
flavored gelatinous mass made of 100 gm of soluble starch and
450 cc of water. Venous blood specimens are taken before, and a
half, one, two, and three hours after the administration of the
starch. To interpret the results properly it is necessary to obtain,
in addition, a glucose tolerance curve, following ingestion of 100 gm
of glucose. The maximal rise in blood sugar during the glucose
and the starch tolerance tests is determined by subtracting the
value for the fasting blood sugar of each curve from that of the
peak of the respective curves. Then, the extent to which the
maximal rise in blood sugar after glucose exceeds that after starch
is calculated in percentages and compared with the normal range
obtained from patients without diseases of the pancreas.

They found that there was no overlapping of the results of »the
starch tolerance test» performed in 27 individuals without pan-
creatic disease and in 23 patients with chronic pancreatic disease.
In 87% of cases in the latter group the outcome of the test was
definitely abnormal (above the mean of the controls plus three
times the standard deviation). Among 10 patients with suspected
chronic pancreatitis the test was definitely abnormal in 60% of
cases. They have also studied a similar test based on the proteolytic
activity of pancreatic juice in the intestine, but this failed to yield
results helpful in the diagnosis of pancreatic disease.

PRESENT INVESTIGATION

In this study the glucose and starch tolerance tests were performed in ethionine induced experimental pancreatitis. The experimental procedures of the tests followed essentially that of the original method described by Althausen and Uyeyama (2). However, we feel it not to be proper to use the term starch tolerance test. As will be pointed out later, the consideration of the glucose tolerance as an essential part of the mathematical treatment and interpretation of the test is necessary.

MATERIAL AND METHODS

13 rabbits weighing 2.3—2.8 kg were used in the work. After a large number of preliminary trials the suitable amounts of glucose and starch to be used were established. The final procedure was as follows.

Glucose and Starch Tolerance Test. — Each test was made after a fasting of 14—15 hours. After this time 3.5 gm/kg of glucose were introduced by a gastric tube in the glucose tolerance test and 8 gm/kg of soluble starch (Pharmakon, Finland) in the starch tolerance test. The glucose was dissolved in 100 cc of warm water. Soluble starch was suspended in 40 cc of water by mixing with a spoon. Just before the test this suspension was poured into 60 cc of water which had just ceased boiling, and was thoroughly stirred. Blood samples of the ear veins were taken before, and a half, one, two, and three hours after administration of the glucose or the starch. The blood sugar determinations were made according to the Hagedorn-Jensen method (10) and the mean of the duplicate samples was recorded.

Calculation of the Results. — This was made similarly with the investigation of Althausen and Uyeyama (2). The maximal rise in blood sugar during the glucose and the starch tolerance test was determined by subtracting the value for the fasting blood sugar of each curve from that of the peak of the respective curves. Then, the extent to which the maximal rise in blood sugar after glucose exceeds that after starch was calculated in percentages of the maximal rise after starch. Althausen and Uyeyama have used the term »starch test %» of this figure. This is perhaps misleading since

the test % depends also upon the changes in the glucose tolerance curve. It is therefore justified to use the term glucose and starch tolerance test %.

Ethionine and Experiments. — DL-ethionine (General Biochemicals, Inc., Ohio, U.S.A.) was dissolved in water with the aid of heat (50°C) to give a solution containing 25 mg/cc. Ethionine was given orally with a gastric tube in daily doses of 25 mg/kg. This dosage was adopted after few experimental trials with larger doses.

In addition to the preliminary experiments in which 8 rabbits were used, the final complete experiments were carried out with 5 rabbits. Each one served as its own control. Thus 3 glucose and 3 starch tolerance tests were performed on each animal before the ethionine treatment. The glucose test was always made first, the starch test following it after 2 days interval. The tests were not repeated earlier than 2 days later. After these control tests were made ethionine was given and continued until the animals died or were sacrified in agone conditions. It was thus possible to perform the glucose and starch tolerance tests in this order in each rabbit on the 4th—6th day and on the 9th—11th day after the beginning of the ethionine treatment. In only one case this period was 13—15 days.

RESULTS

During the ethionine administration each rabbit developed in few days a syndrome characterized by anorexia, loss of weight, jaundice and weakness. In addition they had loose stools and gave evidence of a bleeding tendency by the development of hematomas at sites of venipuncture. Four rabbits died 12—14 days and the fifth 16 days after the beginning of the ethionine feeding.

Fat necrosis of the pancreas was present in the autopsy of all animals. This extended down to the mesenterium, omentum and perirenal fat tissue. The liver had a slight yellowish appearance. Other organs such as the kidney, gastrointestinal tract, heart and lungs showed no significant changes.

Histological specimens[1] were made of the pancreas, liver and

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[1] The authors are indepted to Dr. A. Helminen, acting associate professor of pathology, University of Turku, for his kind assistance in the interpretation of the histological changes.

kidney. The pancreas preparations were characterized by disruption of acinar architecture, focal or diffuse necrosis of the acinar cells, fat necrosis, interstitial infiltration of lymphocytes and poly-morphonuclear leucocytes and fibrosis. A minute focus of hemor-rhagic necrosis was seen in some sections. No lesions of the ducts, blood vessels or islets were seen. These findings correspond nearly those found in subacute pancreatitis.

In the liver were seen no other microscopic abnormalities than the moderate fatty changes. The kidney showed no marked histo-logical changes, only minimal fatty changes in the proximal con-voluted tubules were seen.

Glucose and Starch Tolerance Test. — The results of the glucose and starch tolerance test are shown in table 1. This shows that the

TABLE 1

CALCULATED GLUCOSE AND STARCH TOLERANCE »TEST %» IN FIVE RABBITS BEFORE AND AFTER INTRAGASTRIC ADMINISTRATION OF 25 MG/KG PER DAY OF ETHIONINE. THE CONTROL VALUES REPRESENT THE MEAN OF THREE DETER-MINATIONS

| | Rabbit No. | | | | | Mean |
	1	2	3	4	5	
Control	52	20	19	54	17	32
4—6 days after ethionine	189	289	249	315	153	239
9—11 days after ethionine....	579	632	532	614	374	546
13—15 days after ethionine ..					715	

effect of ethionine on the course of the test is evident in all animals. The mean results obtained 4—6 days after ethionine feeding are 7 times greater than those of the controls. The 9—11 day figure is about 17 higher than the control mean·

Further statistical analysis gives the mean 32 for the control period with a standard deviation of 19. Using these figures and the equation for normal range (mean $\pm$ 2 $\times$ standard deviation) we obtain the range exceeding from — 6 to + 70. Since no negative values were present in these experiments the higher limit for normal range is only of importance. Fig. 1 represents graphically these results. The calculated normal limit 70 is also shown in it.

The average blood sugar curves before and after administration of ethionine, determined by subtracting the value for the fasting blood sugar, are shown in Figs. 2, 3 and 4. These figures show that
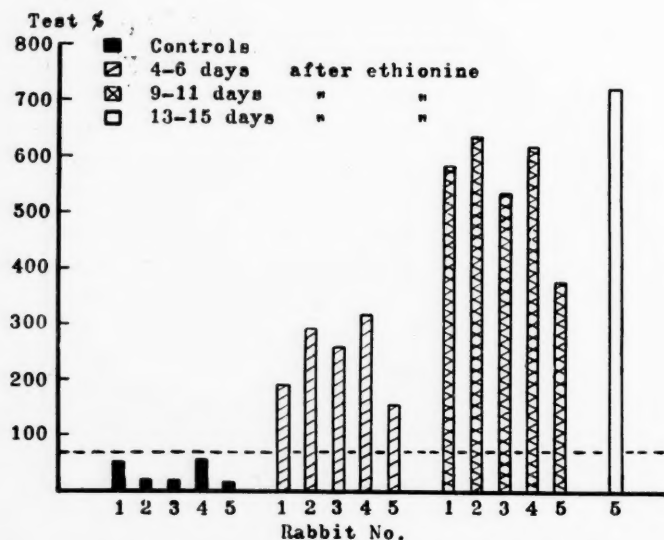
Fig. 1. — Calculated glucose and starch tolerance »test %» in five rabbits before and after intragastric administration of 25 mg/kg per day of ethionine. The control values represent the mean of three determinations. - - - - - calculated normal limit.
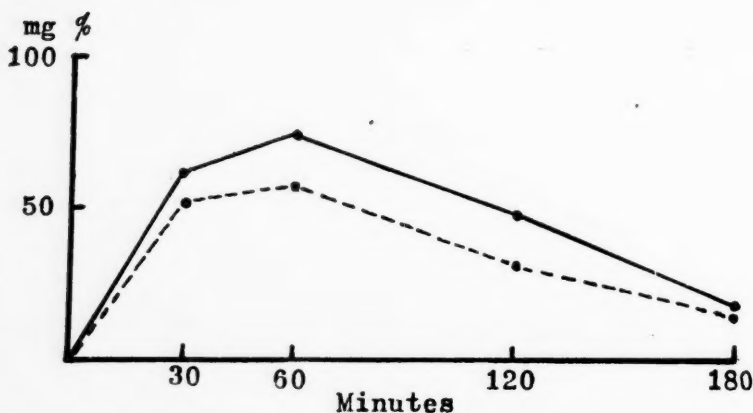
Fig. 4. — The average blood sugar curves in five rabbits after intragastric administration of 25 mg/kg per day of ethionine for 9—11 days, determined by subtracting the values for the fasting blood sugar (which are shown in table 2). ——————— after glucose, - - - - - after starch.
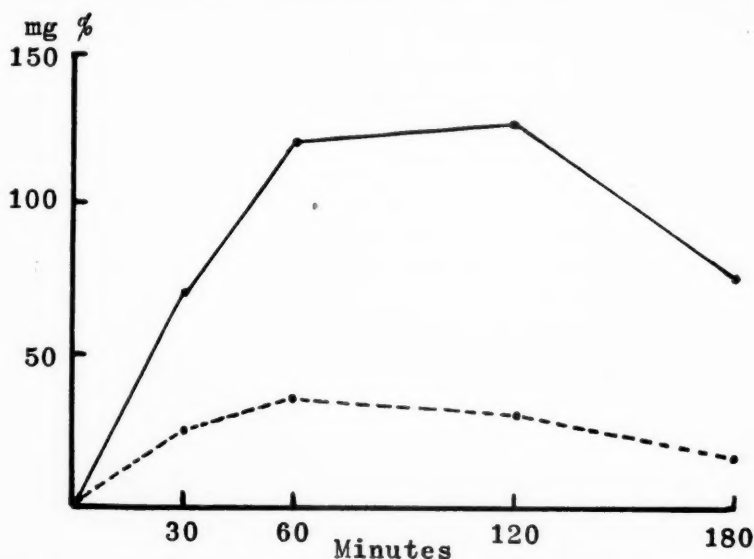
Fig. 3. — The average blood sugar curves in five rabbits after intragastric administration of 25 mg/kg per day of ethionine for 4—6 days, determined by subtracting the values for the fasting blood sugar (which are shown in table 2).
———————— efter glucose, - - - - - - after starch.
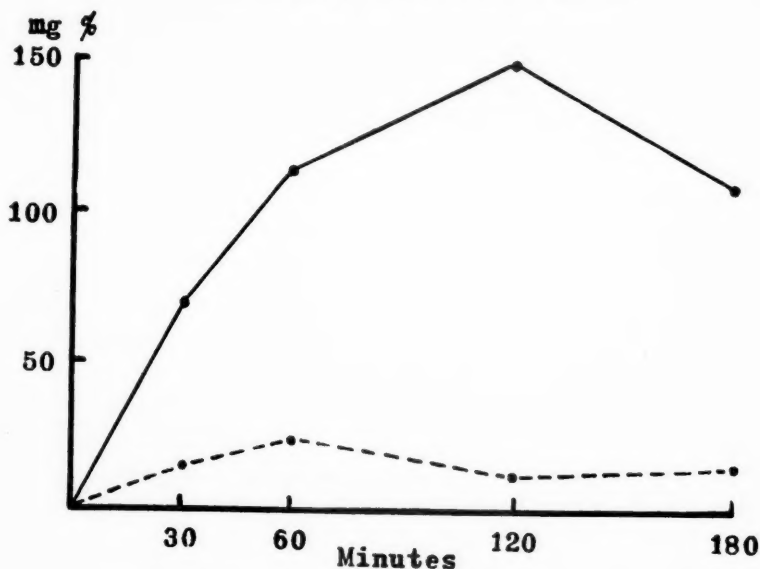
Fig. 2. — The average blood sugar curves in five rabbits before administration of ethionine, determined by subtracting the values for the fasting blood sugar (which are shown in table 2). ———————— after glucose, - - - - - - after starch.

during the progress of ethionine lesions in the pancreas the rise in the glucose curve is approximately of the same magnitude as the lowering of the starch curve. The slope of the glucose curve during the first half hour is about the same order in all figures. The peak of the glucose curve is reached after one hour in figure 2 whereas this same occurs only after two hours in figures 3 and 4.

The initial values for the fasting blood sugar are shown in table 2. They show a slight rising tendency during the ethionine treatment.

TABLE 2

THE INITIAL VALUES FOR THE FASTING BLOOD SUGAR

	Number of Determinations	Range mg %	Mean
Control	30	91—127	111
4—6 days after ethionine	10	104—137	116
9—11 days after ethionine	10	104—135	119
13—15 days after ethionine	2	120—126	123

DISCUSSION

Before discussing the effect of ethionine on the pancreas and the glucose and starch tolerance test, it should be emphasized that ethionine produces not only lesions of the pancreas. This became evident already from described changes in the liver and kidney. Nearly in all previous studies it has been mentioned additional fatty metamorphosis of the liver (1, 3, 6, 7, 8, 9, 11, 13, 16, 17, 19, 20). Fatty changes in the proximal convoluted tubules of the kidney have been described repeatedly (3, 9, 16, 19, 21). In addition, it has been reported the occurrence of changes in the gastrointestinal tract (1, 4, 15), the bile ducts (3, 12, 16), the submaxillary glands (15), the testes (3) and the adrenals (9). At present the action of ethionine can be understood by considering it in part at least as an antimetabolite of its structural analogue, methionine. Ethionine has been shown generally to depress protein synthesis and to inhibit incorporation into protein of methionine and possibly other amino acids (18). The general symptoms in the animals are obviously a consequence of the non specific effects of ethionine.

The pancreatic lesions observed in this work agree with those described previously in connection of ethionine pancreatitis (1, 3,

6, 7, 8, 9, 11, 13, 15, 16, 17, 19, 20). Our standard dose 25 mg/kg daily can be considered as a medium size dose. As mentioned before the amount of the ethionine dose is directly proportional to the severity of the pancreatic lesions. It is of importance to point out that neither in this investigation changes in the Langerhans islets nor the blood vessels in the pancreas were present. In this respect they are in accordance with just mentioned previous studies. In addition, experiments using radioactive methionine (22) have shown that the rapid early turnover of methionine by the pancreas is due to metabolic activity of the exocrine portion of the gland rather than needs of the islets.

Castrini has reported an interesting study of the presence of ethionine induced changes also in the endocrine pancreas (5). In these experiments 3 or 4 days treatment with ethionine was followed by a progressive increase in the islets of cellular elements of the a-type, elements in which the study of the nucleic acids seems to indicate an evident state of incretory activity. Successively there follows a second phase characterized by the signs of a diminished function, while the islets seem to increase in number and volume. At the same time takes place in the liver »a fat degeneration in two phases: the first initial phase, modest and stationary for 3—4 days, characterized by the presence of small quantities of fat at the periphery of the lobule, to which follows another phase of rapid invasion, progressive and complete of the whole hepatic lobule».

Castrini concludes by indexing the fat degeneration caused by ethionine to an indirect mechanism of this amino acid, namely, through the modifications induced in the protein metabolism. Considering, furthermore, that the a-cells would be assigned to the secretion of a particular hormone (lipocaic) with an action on the fat metabolism, the author deduces that in the first phase of the steatogenic action of ethionine there is an apposition by the insular apparatus through an abundant production of lipocaic (numerical increase and evident functional activity of the a-cells) to which follows a state of diminished insular activity, immediately accompanied by the appearance of an evident and diffused liver steatosis.

Our glucose and starch tolerance test results are in some respect in accordance with the findings of Castrini (5). The changes in the glucose tolerance curves during ethionine treatment are signs of

the reduction of glucose tolerance. The slight elevation of the
initial values for the fasting blood sugar during the administration
of ethionine as presented in table 2 also suggests this. Our studies
therefore point to the probability that ethionine also has an effect
on the endocrine function of the pancreas. Of course the possible
role of changes in the liver and adrenal functions must be taken
into consideration in this connection.

Nevertheless, the specific effect of pancreatic changes on the
course of the test seems be very clear as regards to the starch
tolerance curves. The less rise in the curves can be taken as an
indication of a great reduction in the production of amylase in the
pancreatic juice due to the production of pancreatitis by ethionine.
This effect exceeds in severity the possible insufficiency in the
endocrine function of the pancreas. That the absorption of glucose,
both of the given as that formed from starch, would be disturbed,
seems inplausible. The mean slope of the glucose tolerance curves
during the first half hour period remained the same magnitude
even after the treatment with ethionine.

Comparison of our observations with the corresponding experi-
ments on patients suffering from chronic pancreatitis (2) reveals
clear resemblance in the results. The mean blood sugar curves
particularly resemble each other since also in the investigation of
Althausen and Uyeyama the glucose tolerance curves rises in the
cases of pancreatitis about in the rate as the starch tolerance curves
has lowered. The term »starch tolerance test» does not seem to be
tenable in light of these findings.

On basis of all above mentioned the conclusion might be made
that the effect of ethionine on the course of the glucose and starch
tolerance test lies mainly if not solely on the changes in pancreas.
These observations also strongly support the investigation of
Althausen and Uyeyama and suggest the suitability of the glucose
and starch tolerance test in the studies of the pancreatic functions.

SUMMARY

Ethionine, the ethyl analogue and antimetabolite of methionine,
was given to rabbits this resulting in the usual production of sub-
acute pancreatitis with accompanying moderate fatty metamor-
phosis of the liver. Pancreatitis was characterized by disruption

of acinar architecture, focal or diffuse necrosis of the acinar cells, interstitial inflammatory reaction, fat necrosis and fibrosis. No lesions of the ducts, blood vessels or islets were seen.

Glucose and starch tolerance tests were performed on rabbits before and after the ethionine treatment. The results were treated in following manner: The maximal rise in blood sugar during the glucose and the starch tolerance test was determined by subtracting the value for the fasting blood sugar of each curve from that of the peak of the respective curves. Then, the extent to which the maximal rise in blood sugar after glucose exceeds that after starch was calculated in percentages of the maximal rise after starch. The test % calculated by this means revealed a significant rise after ethionine treatment. The mean test % during the control period was 32, standard deviation being 19. The normal range calculated from these figures lies between —6 and +70. The mean results after 4—6 days treatment with ethionine was 239, after 9—11 days it was 546.

The mean slope of the glucose tolerance curves remained unchanged during the first half hour before and after ethionine treatment. This suggests an undisturbed absorption of glucose. The continued rise in the glucose tolerance test with a marked lowering of the starch tolerance curve is a characteristic feature for the period of ethionine treatment. These findings are taken as an indication that in addition to the lowering of the amylolytic activity of the pancreas an insufficiency of the endocrine function of the pancreas is present.

Our results are similar to those reported by Althausen and Uyeyama in patients suffering from chronic pancreatitis. They also lend support to their claim of the suitability of this test for studies of the exocrine function of the pancreas.

REFERENCES

1. DE ALMEIDA, A. L., and GROSSMAN, M. I.: Gastroenterology. 1952:*20:* 554.
2. ALTHAUSEN, T. L., and UYEYAMA, K.: Ann. Int. Med. 1954:*41:*563.
3. ALVIZOURI, M., and WARREN, S.: A. M. A. Arch. Path. 1954:*57:*130.
4. CASTRINI, G.: Riv. di Biol. 1954:*46:*489.
5. CASTRINI, G.: Ibidem. 1954:*46:*481.
6. FARBER, E., and POPPER, H.: Proc. Soc. Exper. Biol. & Med. 1950: *74:*838.

7. FITZGERALD, P. J., and ALVIZOURI, M.: Nature, London. 1952:*170*:929. Quoted by LORING and HARTLEY.
8. GOLDBERG, R. C., CHAIKOFF, I. L., and DODGE, A. H.: Proc. Soc. Exper. Biol. & Med. 1950:*74*:869.
9. GOLDBERG, R. C., and CHAIKOFF, I. L.: A. M. A. Arch. Path. 1951: *52*:230.
10. HAGEDORN, H. C., and JENSEN, B. N.: Biochem. Z. 1923:*135*:46.
11. KALSER, M. H., and GROSSMAN, M. I.: Gastroenterology. 1954:*26*:189.
12. KOCH-WESER, D., and POPPER, H.: Proc. Soc. Exper. Biol. & Med. 1952:*79*:34.
13. KROBOTH, F. J., and HALLENBECK, G. A.: Gastroenterology. 1954: *27*:743.
14. LIN, T. M., and GROSSMAN, M. I.: Am. J. Physiol. 1954:*176*:377.
15. LORING, W. E., and HARTLEY, L. J.: Am. J. Path. 1955:*31*:521.
16. POPPER, H., DE LA HUERGA, J., and KOCH-WESER, D.: (Abstract.) Ibidem. 1952:*28*:518.
17. POPPER, H., and DE LA HUERGA, J.: Proc. Inst. Med. Chicago. 1952 —53:*19*:152. Quoted by LORING and HARTLEY.
18. SIMPSON, M. V., FABER, E., and TARVER, H.: J. Biol. Chem. 1950: *182*:81.
19. WACHSTEIN, M., and MEISEL, E.: Proc. Soc. Exper. Biol. & Med. 1953:*82*:70.
20. WACHSTEIN, M., and MEISEL, E.: Lab. Investigation. 1953:*2*:253.
21. WACHSTEIN, M., and MEISEL, E.: Proc. Soc. Exper. Biol. & Med. 1951:*77*:648.
22. WHEELER, J. E., LUKENS, F. D. W., and GYÖRGY, P.: Ibidem. 1949: *70*:187.

THE EFFECTS OF TRAINING ON HEART RATE

A »LONGITUDINAL» STUDY

by

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Training is known to lower the heart rate. Both the resting heart rate and the heart rate at a standard exercise become slower. This has been observed by comparing the heart rates of athletes and non-athletes, as well as in a few »longitudinal» studies, in which the heart rate of the same subjects has been observed during training (e.g. 6).

The maximum heart rate attainable during exercise is known to depend on age and sex (2). The maximum rate is claimed to be also dependent on physical fitness: it is less in athletes than in non-athletes (14). Other investigators have come to a contrary result; according to them, there is no difference between the maximum heart rates of trained and untrained subjects (12, 16, 19). In a longitudinal study (18) the maximum heart rate actually became slower during training.

In the present study, training of different intensities was used, and its effect on the resting, working and maximum heart rates was studied. The purpose was to find out what kind of quantitative relations may prevail between the intensity of training and the heart rates.

MATERIAL AND METHOD

The *subjects* were six male medical students, age from 20 to 23 years. One of them performed two training experiments, with an interval of five weeks. The subjects trained by *running* on a horizontal treadmill. The time run each day was constant, 30 minutes. Training occurred during 4 or 5 days a week, and each training experiment lasted 4 weeks.

The *working heart rate* (WR) was counted by using a stethoscope; the subject stepped off the treadmill for appr. 15 seconds, and a 10 sec. pulse count was taken. This was repeated every 10 minutes. Such a procedure is known to give a good approximation of the pulse rate during actual work (10, 17). The mean of the three counts is given as WR.

The aim was to keep the intensity of training constant during each training period. For this purpose, the *speed of running* was adjusted so that WR remained as close as possible to the predetermined level.

Each subject counted his *resting heart rate* (RR) every morning during the training period, in bed, before getting up.

The *maximum running heart rate* (MR) was determined at the beginning and at the end of each training period. The subjects ran at a submaximal speed until exhaustion, which with the speeds used was reached in 2 minutes or less; the heart rate was counted in the same way as in determining WR.

A chest roentgenogram was taken at the beginning and end of each training period, for determining the *heart volume* (22).

RESULTS

The results of the training experiments are shown in Table 1. A decrease of WR as effected by training appears in these experiments as an increase of the running speed. The intensity of training is expressed in two ways: (1) as WR, and (2) as $\frac{WR}{MR-RR} \times 100$; this expresses the WR as a percentage of the total range of pulse rates from rest to the maximum attainable by running, as calculated from the MR and RR at the start of training. The first value of RR was used as the beginning figure. For the

TABLE

SUMMARY OF THE RESULTS OF TRAINING EXPERIMENTS, IN THE ORDER OF INCREASING INTENSITY OF THE TRAINING.

WR = working heart rate, MR = maximum heart rate, RR = resting (morning) heart rate

Subject	Runing Speed km/hr			Mean WR	WR / MR-RR×100	MR			RR			Heart Size cc		
	Begin	End	Δ			Begin	End	Δ	Begin	End	Δ	Begin	End	Δ
O.M. (1) ..	9	9	0	135	60	180	180	— 0	53	51	—2	560	585	+ 25
E.K.	9	9	0	136	60	192	186	— 6	52	48	—4	655	790	+135
K.L.	9	10	+1	137	60	192	174	—18	55	52	—3	525	780	+255
E.H.	11	13	+2	135	71	170	156	—14	52	46	—6	760	715	— 45[1]
O.M. (2) ..	11	13	+2	150	75	186	174	—12	55	50	—5	555	560	+ 5
O.K.	11	14	+3	160	75	192	186	— 6	58	55	—3	670	665	— 5
J. K.	11	14	+3	180	74	224	184	—40	52	48	—4	820	665	—155[1]

[1] Heart volume was measured from the orthodiagram only, using Bardeen's formula (5).
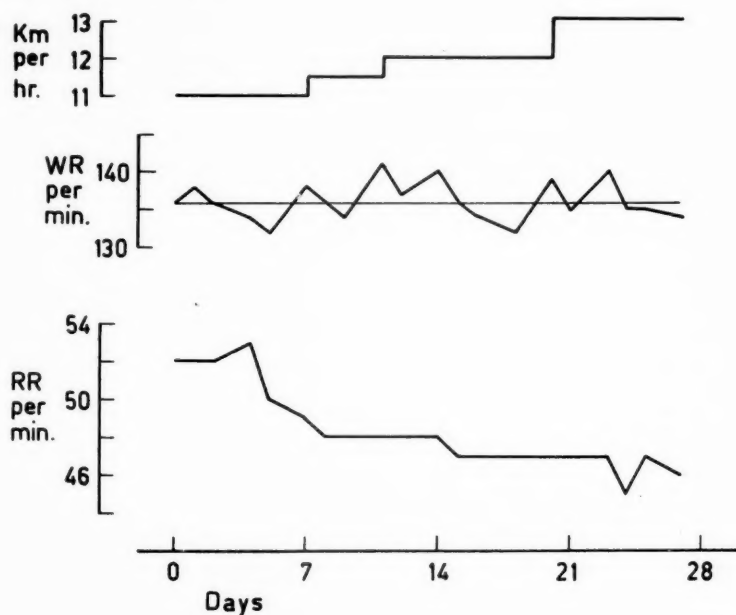
Fig. 1. — Speed of running (km per hr.), running heart rate (WR), and resting heart rate (RR) of subject E. H. during a training period. Running at the heart rate 136 per min. which was 71 per cent of the available range of heart rates at the beginning, produced a training effect which necessitated a progressive increase of the running speed.

ending figure the lowest pulse rate counted at least on two mornings was chosen; generally the lowest RR:s were observed at the end of the training period.

As an example, the results of an experiment are shown in detail in Fig. 1.

The results indicate that training in some experiments caused a decrease of WR which necessitated an increase of running speed, but in others no change of WR occurred. A decrease of WR was observable in those experiments in which running was fastest. If WR was used as an indicator of the intensity of training, the borderline between »effective» and »ineffective» training was not sharp. However, if the WR was expressed as a percentage of the range of pulse available, there appeared to be a critical limit at or slightly above the 60 per cent training level.

The MR decreased, in all but one of the experiments, but the decrease of MR apparently was not related to changes in WR or RR. The greatest fall, 40 beats per min., was observed in the subject who had the highest MR to start with.

The RR fell a few beats in every series. The extent of the decrease was not related to changes in WR or RR.

The roentgenological volumes of the heart showed both increases and decreases, with no clear relation to the intensity of training.

DISCUSSION

It is well known that training can cause a decrease of the WR, and several types of tests of »physical fitness» are based on counting the exercise or post-exercise heart rate. The work of Åstrand (2) and Ryhming (20) shows that there is a close correlation between the maximum oxygen uptake of a subject and his or her heart rate response to graded exercise, which correlation may be used as a basis for assessing the maximum oxygen uptake from the heart rate recorded during submaximal work; a nomogram for this purpose has been published (4).

The most interesting result of the present study is that consider-able time and effort may be devoted to training at a work and heart rate level distinctly above those customary in medical studies, without the actual benefit of a measurable decrease of the WR. If the correlation between WR and maximum oxygen uptake is

to be accepted also in this situation, the training has been without effect also on the latter. On the other hand, if training occurs at a more intense level, with pulse rates above 150 per min., or taxing at least 70 per cent of the available range of pulse rates, the training will cause a decrease of the WR.

These findings have an important bearing on the planning of athletic training. In order to cause an increase of the maximum oxygen uptake, training must be intense. Misguided philanthropism in the administration of athletic training programs may deprive them of one of their major effects.

In the physiological rationalisation of occupational work, normative limits have been defined, above which work is considered to be unduly heavy for the human organism. One of these norms is that in work done for a full working day, the heart rate ought not to rise above 125 or 130 per minute. In relatively short operations, heart rates of up to 150 are to be allowed (8).

The normative limit for heart rate at continuous work is distinctly below those values at which a training effect on WR was obtained in the present series. According to experience from a number of occupations heart rates above 130 per minute are relatively rare in industry. The present tendency in industry is to more uniform work, and to an exclusion of physically heavy operations from the day's program. These factors make it obvious that occupational work, even relatively heavy physical work, rarely exerts a training effect on the heart, i.e. a decrease of the WR. If the worker wishes to increase his cardiovascular reserves — as expressed by the margin above the level of oxygen uptake required in his particular work — he has to take recourse to activities more intense than those offered by his occupation.

Running on a level course may not be the most practical and efficient means for an improvement of the WR and of the maximum oxygen uptake. In activities like skiing, in which larger groups of muscles participate, higher MR:s and maximum oxygen uptakes have been obtained than in running (9,23). Heart rates which in the present study proved sufficient to produce a training effect on the WR, are — according to the writers' experience — subjectively more easily attained by skiing than by running.

MR and RR decreased during training in all experiments, without evidence for the need of a similar critical limit in the

intensity of training than with WR. Johnson and Brouha (14) report distinct differences in MR according to the state of training and physical fitness, in the same sense as those observed in the present study. Montoye, in a longitudinal study of changes in MR during a 12 weeks' training period, observed a mean decrease by 15 beats per min. in 50 subjects (18).

The factors governing the effects of training on the heart rate at rest and in work are multiple. The increase of the heart rate during work is understood probably occurring as a reflex response, in which the afferent stimuli come from the working muscles (1). It is reasonable to assume that the impulse drive from the muscles will become particularly intense when the conditions in the muscle become anaerobic. Training improves the blood supply of working muscles locally. Anaerobic conditions are not as easily produced in a trained muscle as in an untrained one. This reasoning is in line with the observation that the maximum pulse rates attained by running decreased during training: the afferent impulse drive from the trained muscles during maximum effort appears to have been less than at the beginning of training.

In work at a steady state, the muscles work under predominantly aerobic conditions. If anaerobiosis is important in determining the impulse drive from the muscles, as assumed above, training may be expected to have less effect on heart rates at »steady state» levels than in more intense work in which products of anaerobic metabolism pile up in the working muscles. This reasoning is in agreement with the present results, in which MR could decrease considerably, without a parallel change in WR. The WR probably depends relatively more on the condition of the heart and less on that of the peripheral muscles.

Previous experience has shown that among various indicators of exercise tolerance, the recovery of the heart rate after exercise is sensitively correlated with the amount and intensity of training (e.g. Karvonen: unpublished observations). The rate of recovery is used as a basis for several tests of cardiovascular fitness. At relatively low work intensities, at which the concentration of anaerobic metabolites in the circulating blood remains low, the rate of recovery probably depends on the local vascular conditions of the working muscles. At high intensities of exercise anaerobic metabolites, notably lactic acid, are present in blood at high con-

centrations. In such circumstances, the rate of recovery of the heart rate is an indication also of the adequacy of the cardiac output during work. In the present study the rate of recovery was not determined.

It is generally agreed that variations of RR are to a large extent irrelevant for cardiovascular fitness: adaptation to work is not strictly dependent on the type of adaptation to rest. The independence of RR from WR and MR is in agreement with such a conclusion.

Great individual differences certainly exist in the training process. The importance of individual differences is suggested e.g. by the irregular changes in the roentgenological volume of the heart in the present series. Although a pronounced positive statistical correlation prevails between maximum oxygen uptake, total blood volume and resting heart volume (2, 15), training of moderate intensity may in an individual subject lead to a change even in the opposite direction, as in the subjects E. H. and J. K.

The present study deals with only one of the variables in endurance training, the speed of running. The time of each training run, the frequency of the runs, and the total duration of the training period were kept constant. All these factors, and, moreover, the physical fitness of the subjects at the beginning of the training period may be important determinants of the results of training. The effects of each of these factors has to be ascertained separately. However, the rate of work rather than the total amount of it probably is the most important factor in training (7, 11, 13, 21).

The method of training used in the present study makes it possible to keep the training physiologically constant, at a predetermined heart rate level, and to gauge simultaneously the effect of training on an important component of exercise tolerance, the maximum oxygen uptake, from the adjustments of speed necessary for keeping the WR constant. A further advantage of the method is that the results are quite independent of the motivation of the subjects, since training occurs and all essential observations are made at the level of submaximal effort.

SUMMARY

Six young male subjects trained by running on treadmill half an hour daily, 4 to 5 times a week, over a period of 4 weeks; one of the subjects did two training periods. The speed of the treadmill was adjusted so that the working heart rate (WR) remained at a pre-determined level throughout the training period.

Training by running at a relatively high speed, and consequently with a high heart rate, caused a decrease of the WR, which was counteracted by further increasing the speed of running. However, running at a slow speed and thus with a low heart rate did not produce any decrease of the WR. It was concluded that the heart rate during training has to be more than 60 per cent of the available range from rest to the maximum attainable by running — or above appr. 140 per minute — in order to produce a decrease of the WR. A decrease of the WR is understood to indicate an increase of the maximum oxygen uptake. Training by running at lower speeds and pulse rates may nevertheless cause a decrease of the resting heart rate (RR) and of the maximum heart rate (MR) attainable by running. Changes of RR, MR and WR were largerly independent of each other; those of RR and MR also from the intensity of the training.

The bearing of the results on athletic training and on occupational work is discussed.

REFERENCES

1. Asmussen, E., and Nielsen, M.: Physiol. Rev. 1955:35:778.
2. Åstrand, P.-O.: Experimental studies of physical working capacity in relation to sex and age. Copenhagen 1952.
3. Åstrand, P.-O.: Physiol. Rev. 1956:36:307.
4. Åstrand, P.-O., and Ryhming, I.: J. appl. Physiol. 1954:7:218.
5. Bardeen, C. R.: Amer. J. Anat. 1918:23:423.
6. Christensen, E. H.: Arbeitsphysiol. 1931:4:453.
7. Christensen, E. H.: Arbeitsphysiol. 1931:4:470.
8. Christensen, E. H.: In Ergonomics Society Symposium on Fatigue. Pp. 93—108. London 1953.
9. Christensen, E. H., and Högberg, P.: Arbeitsphysiol. 1950:14:292.
10. Cotton, F. S., and Dill, D. B.: Amer. J. Physiol. 1935:111:554.
11. De Lorme, J.: J. Bone Jt Surg. New Ser 27, 1945:43:645.
12. Dill, D. B., and Brouha, L.: Travail hum. 1937:5:3. Ref. by Åstrand (3).

13. Edwards, H. T., Brouha, L., and Johnson, R. E.: Travail hum. 1940:*8:*1. Ref. by Åstrand (3).
14. Johnson, R. E., and Brouha, L.: Rev. Canad. Biol. 1942:*1:*171.
15. Kjellberg, S. R., Rudhe, U., and Sjöstrand, T.: Acta physiol. scand. 1949:*19:*152.
16. Knehr, C. A., Dill, D. B., and Neufeld, W.: Amer. J. Physiol. 1942:*136:*148.
17. Lundgren, N.: Acta physiol. scand. 1946:*13:*41.
18. Montoye, H. J.: Research Quarterly 1953:*4:*453.
19. Robinson, S., Edwards, H. T. and Dill, D. B.: Science 1937:*85:*409.
20. Ryhming, I.: Arbeitsphysiol. 1954:*15:*235.
21. Siebert, W. W.: Z. klin. Med. 1929:*109:*350.
22. Strandquist, M.: Acta radiol. ,Stockh. 1935:*16:*304.
23. Taylor, H. L., Buskirk, E., and Henschel, A.: J. appl. Physiol. 1955:*8:*73.

EFFECT OF CHLORPROMAZINE AND RESERPINE ON LIVER PARENCHYME IN THE WHITE RAT

by

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In the psychiatric chlorpromazine treatment jaundice occurs as a complication in 1—2 per cent of cases (3, 7, 8). It manifests itself mostly within 2—3 weeks from the beginning of the treatment and is considered to be caused by an intrahepatic obstruction. The occurrence of jauncide is rather independent of the dosage of chlorpromazine. It has been assumed that it here is a question of an allergic phenomenon, although its mechanism has not been completely clarified (4, 14). In the biopsies it has been found bile thrombi in the finest bile ducts but in the liver parenchyme it has been found only little or not at all changes (19, 21, 22). During long time chlorpromazine treatment, independent of this chlorpromazine jaundice, it has sometimes been presented in the liver function tests alterations pointing to parenchyme lesion. Especially in cases with a primary insufficiency of the liver function the liver function tests have proved to be impaired during chlorpromazine treatment (10, 15, 16, 23). The clinical observations, however, are contradictory in this respect (12, 13, 17, 18).

In laboratory animals it has been found that a short time chlorpromazine medication does not even in large doses damage the liver parenchyme (5). Chlorpromazine has not been found, too, to have any depressing effect on the tissue culture respiration of the rat liver (20). On the other hand, it has not been performed

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experiments concerning the possible effect of a long time chlor-promazine medication to the liver parenchyme. In the psychiatric use of chlorpromazine, however, it often is just a question of long time treatment. The purpose of the following study is to search out if chlorpromazine causes any changes in the liver parenchyme and of what kind the changes are in that case. For comparison experimental series were performed also with reserpine that has not been found to affect the liver function in the clinical use (6, 9, 11) and with combined chlorpromazine-reserpine medication. These are methods of treatment that together with chlorpromazine have actual interest in psychiatry.

<h2>MATERIAL AND METHODS</h2>

The effects of large doses of chlorpromazine and reserpine were studied by three groups of female rats of the same age. Reserpine was administrated daily to the 29 rats of the first group 0.75 mg/kg body weight, to the 10 rats of the second group 0.45 mg/kg body weight in addition to chlorpromazine 30 mg/kg body weight. To the 10 rats of the third group was given chlor-promazine 50 mg/kg body weight daily. The drugs were applied subcutaneously. The medication was continued until death. In the second series the daily dosages were increased slowly for that the animals would adapt themselves to the medication. To the 9 rats of the first group was given reserpine 0.075 mg/kg body weight subcutaneously on the first day, to the 9 rats of the second group reserpine 0.025 mg and chlorpromazine 5 mg/kg body weight and to the 18 rats of the third group chlorpromazine 5 mg/kg body weight. The dosage was increased daily with the dose of the first day in each group. The medication was continued during 25 days, after this the rats still alive were killed.

Each rat was weighed before the medication was started and again after death, also the liver was weighed then. To demonstrate the possible changes of the weight of the liver during the medication the body and liver were weighed by 9 control rats. A specimen of the liver for microscopic study was taken as soon as possible after death. Therefore, rats being dying were killed. The possible occur-rence of fat was studied by cutting 10 μ frozen sections and by staining them with the Sudan III method. After this the specimen

was embedded in paraffin and 5 μ sections were cut and stained with the van Gieson method. For glycogen sections were stained also with the Best's carmine method.

<div align="center">RESULTS</div>

The group of 29 rats receiving large doses of reserpine from the very beginning of medication (0.75 mg/kg body weight) died on an average 18 days after the beginning of medication. The body weight was decreased on an average by 29%. In one case fat infiltration of the liver could be seen, in the other 28 cases the liver parenchyme proved to be normal. The group of 10 rats receiving combined medication with large doses of reserpine (0.45 mg/kg body weight) and chlorpromazine (30 mg/kg body weight) died on an average 9½ days after the beginning of medication. The body weight was decreased on an average by 20%. The liver parenchyme proved to be normal in each case. The group of 10 rats receiving large doses of chlorpromazine from the very beginning of medication (50 mg/kg body weight) died on an average 7 days after the beginning of medication. The body weight was decreased on an average by 16%. The liver parenchyme proved to be normal in each case. The group of 9 rats receiving increasing doses of reserpine (the dose being increased by 0.075 mg/kg body weight daily) died on an average 16 days after the beginning of medication. The body weight was decreased on an average by 28%. The liver parenchyme proved to be normal in each case. The group of 9 rats receiving increasing doses of reserpine (increased by 0.025 mg/kg body weight) and chlorpromazine (increased by 5 mg/kg body weight daily) died on an average 15.5 days after the beginning of medication. The body weight was decreased on an average by 35%. The liver parenchyme proved to be normal in each case. The group of 18 rats receiving increasing doses of chlorpromazine (increased by 5 mg/kg body weight daily) became adapted to the medication and tolerated it well. The rats were killed after 25 days of medication, when the dosage was at the level of 125 mg/kg body weight daily. The body weight was de creased on an average by 16%. Fat infiltration of the liver was obtained in 14 cases, necrosis or other regressive changes in 10 cases. Only in one case the histologic picture of the liver proved to be
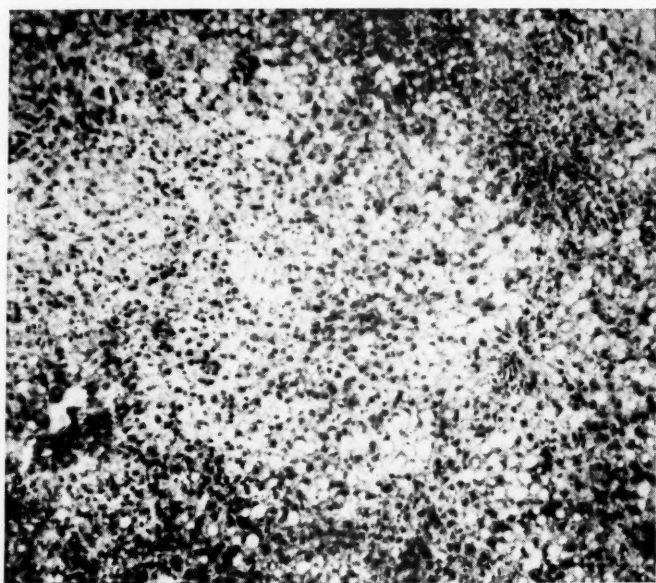
Fig. 1. — Photomicrograph of the rat liver showing extensive fat infiltration caused by prolonged, massive chlorpromazine medication. Van Gieson stain, × 180.
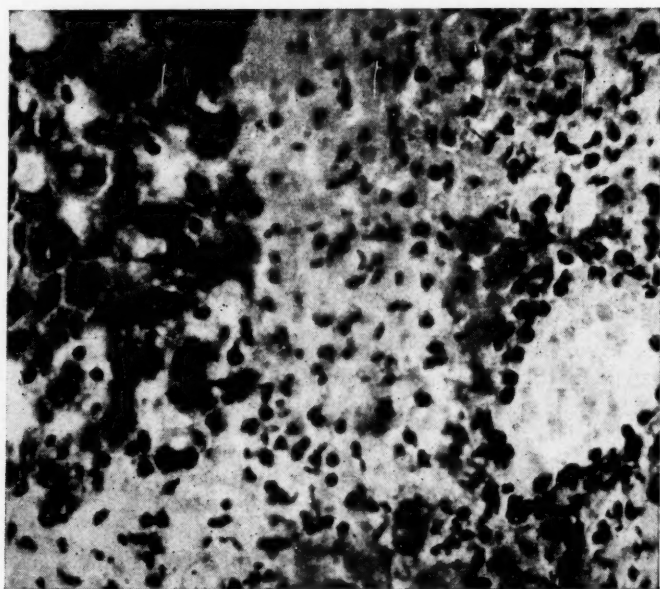
Fig. 2. — Photomicrograph of the rat liver showing necrosis caused by prolonged, massive chlorpromazine medication. Van Gieson stain, × 710.

normal. In this group, the weight of the liver had increased on an average by a fourth during the medication, in the reserpine and combined reserpine-chlorpromazine groups, on the contrary, the weight of the liver had somewhat decreased, by up to a fourth in the group of increasing combined reserpine-chlorpromazine medication.

In the group of increasing chlorpromazine medication, no correlation could be shown between the liver parenchyme damages and the decrease of the body weight or the increase of the weight of the liver. In the cases of this group, following changes could be seen by studying the liver microscopically:

Case 1. — Extensive fat infiltration is seen. The fat droplets, staining positively with Sudan III, are large, filling the cell. In the regions of fat infiltration the glycogen content of the tissue is markedly decreased.

Case 2. — Here and there some single, rather large Sudan positive fat droplets are obtained.

Case 3. — Plenty of Sudan positive fat droplets are seen in the center of the lobules. The fat droplets are large, filling the cell. Everywhere the liver cells are showing degenerative changes: the cytoplasm has lost its structure and partially disappeared so that many cells seem to be almost empty. The cell outlines, however, are distinct and the nuclei well preserved. The glycogen content of the cells is rather low.

Case 4. — Here and there some single, rather large Sudan positive fat droplets are seen, and everywhere in the liver cells there is to be seen regressive changes of the same type as in the case 3.

Case 5. — Large and distinctly outlined necrotic regions are seen at the surroundings of the central veins. On the necrotic regions the glycogen content is very low.

Case 6. — Here and there some single Sudan positive fat droplets are seen and everywhere in the liver cells it is to be seen regressive changes of the same type as in the case 3.

Case 7. — Extensive fat infiltration at the periphery of the lobules is seen. The fat droplets are mostly large, filling the cell and giving a positive reaction with Sudan III. The glycogen content of the tissue, however, is rather high.

Case 8. — Moderate fat infiltration is seen, the fat droplets giving a positive reaction with Sudan III. Everywhere in the liver cells it is to be seen regressive changes of the same type as in the case 3.

Case 9. — In some cells it is to be seen plenty of small Sudan positive fat droplets. In other respects the tissue is normal.

Case 10. — Here and there some single, large Sudan positive fat droplets are seen, and in many liver cells there are plenty of small fat droplets, in some cases almost filling the cell.

Case 11. — In some cells small fat droplets are seen.

Case 12. — Here and there some single, large Sudan positive fat droplets are obtained.

Case 13. — Rather diffusely outlined necrotic regions are seen. They seem to be situated at the surroundings of the central veins. In addition, it is to be seen some fat infiltration and in many cells several smaller fat droplets, in some cases filling the cell.

Case 14. — In some cells plenty of small fat droplets are seen that even may fill the cell. Near the capsule one can see a small necrotic region. The glycogen content of the tissue is rather low.

Case 15. — A rather extensive fat infiltration is seen. The glycogen content of the tissue is rather low.

Case 16. — In some cells considerably of small Sudan positive fat droplets are obtained. Here and there it is to be seen some larger fat droplets filling the cell. In addition, it is seen in the section two small necrotic regions, both situating at the surroundings of the central veins.

Case 17. — Extensive fat infiltration is seen. Below the capsule it is to be seen a large region that is partly formed by plasma cell infiltration, partly by proliferated connective tissue that is sending strands to the interior of the liver parenchyme.

Case 18. — Rather extensive necrotic regions are seen. They are situated mainly at the surroundings of the central veins. Fat could not be demonstrated by the Sudan III method. The glycogen content of the tissue is very low.

DISCUSSION

The results exposed above show clearly that a prolonged, massive chlorpromazine medication in the white rat causes parenchyme lesions of the liver, namely fat infiltration, necrosis and other regressive changes. These lesions were not seen in those animals, who were exposed to so large chlorpromazine doses from the very beginning of the medication that they died of it in a short time.

In the light of these observations it is evident that the prolonged chlorpromazine medication causes a very considerable stress to the liver. The increase of the weight of the liver seen in this group of test animals might be understood as a functional hypertrophy.

The most prominent feature in the microscopic picture was the fat infiltration. This awakens the question of the significance of a possible disturbance in the function of the lipotropic factors in the etiology of these chlorpromazine-caused damages of the liver (1, 2). Further investigation of this question is to be done.

In the reserpine groups only in one case same kind of changes were observed. It thus seems to be justified to conclude that reserpine does not in any essential degree stress the liver. This is in accordance with clinical observations. Also in the groups of combined reserpine-chlorpromazine medication the histologic picture of the liver proved to be normal. If reserpine here had a diminishing effect upon the influence of chlorpromazine cannot be safely decided on the basis of these experiments.

SUMMARY

The effect of chlorpromazine and reserpine on liver parenchyme in the white rat was studied my means of two experimental series. Neither reserpine nor combined reserpine-chlorpromazine could be shown to affect the liver. A prolonged massive chlorpromazine medication, on the contrary, caused considerable damages of the liver. Fat infiltration was the dominating feature in the histologic picture of this chlorpromazine influence. Besides this, necrosis and other regressive changes could be seen. These changes could not be seen after a short time medication with letal chlorpromazine doses. The hypothesis of the significance of an insufficiency in the function of the lipotropic factors in the etiology of these damages is presented.

REFERENCES

1. BEST, C.: Lancet 1934:*1*:1155, 1216, 1274.
2. BEST, C.: Science 1941:*94*:524.
3. COHEN, I.: Am. J. Med. Sc. 1955:*229*:355.
4. COHEN, I., and ARCHER, J.: J. A. M A. 1955:*159*:99.
5. COURVOISIER, S., FOURNEL, J., DUCROT, R., KOLSKY, M., and KOETSCHET, P.: Arch. int. Pharmacodyn. 1953:*92*:305.
6. DELAY, J., DENIKER, P., TARDIEU, Y., and LEMPERIERE, T.: Pr. Méd. 1955:*63*:663.
7. DENBER, H.: J. A. M. A. 1955:*158*:586.

8. DENBER, H., and BIRD, E.: Am. J. Psychiat. 1955:*112:*465.

9. GELLER, W., and MAPPES, C.: Ärztl. Wchnschr. 1956:*11:*413.

10. HORMIA, A.: Acta Psychiat. Neurol. Scand. 1956:*31:*437.

11. HUCHTEMANN, K., and PFLUGFELDER, G.: Schweiz. Med. Wchnschr. 1955:*85:*627.

12. KINROSS-WRIGHT, V.: Am. J. Psychiat. 1955:*111:*907.

13. KINROSS-WRIGHT, V.: Dis Nerv. Syst. 1955:*14:*114.

14. KINROSS-WRIGHT, V.: Psychiat. Res. Rep. 1955:*1:*53.

15. LEHMANN, H.: Canad. M. A. J. 1955:*72:*91.

16. LEHMANN, H., and HANRAHAN, G.: Arch. Neurol. Psychiat. 1954: *71:*227.

17. LOMAS, J., BOARDMAN, R., and MARKOWE, M.: Lancet 1955:*1:*1144.

18. MOYER, J., KENT, B., KNIGHT, R., MORRIS, G., HUGGINS, R., and HANDLEY, C.: Am. J. Med. Sc. 1954:*227:*283.

19. OMMEN, R. v., and BROWN, C.: J. A. M. A. 1955:*157:*321.

20. PERUZZO, L., and FORNI, R.: Pr. Méd. 1953:*61:*1463.

21. POLLACK, B.: Psychiat. Quart. 1955:*29:*439.

22. STACEY, C., AZIMA, H., HUESTIS, D., HOWLETT, J., and HOFFMAN, M.: Canad. M. A. J. 1955:*73:*386.

23. WEBB, R.: M. J. Austr. 1955:*85:*627.

FROM THE KUMPULA STATE HOSPITAL FOR VENEREAL DISEASES, UNIVERSITY OF HELSINKI

IS THE PLACENTA OF A SYPHILITIC MOTHER MORE READILY TRAVERSED BY THE COMPLEMENT-FIXING ANTIBODY THAN BY THE FLOCCULATING ANTIBODY?

by

TAUNO PUTKONEN, ALI MUROMA and AILI VEHMAS

The infants of syphilitic mothers are often seropositive for some time after birth although they later prove to be healthy. These infants have escaped treponemal infection, but the maternal reagins have traversed the placenta into the foetal circulation, from which they disappear after a few months. According to Moore (1), the complement fixation reactions of such infants were more frequently positive (113 of 292 infants) than the flocculation reactions (8 of 158 infants in another group). Moore was not able to account for this difference.

On the basis of Wiener's (2) studies two varieties of antibody are produced in the Rh-sensitized mother, the univalent antibody, which readily passes through the placenta into the foetal circulation, and the bivalent antibody, which for the most part is retained by the intact placenta. A similar separation of antibodies was applied by him to serological tests for syphilis: he stated that the univalent reagin, readily traversing the placenta, is detected by complement fixation, and the bivalent reagin, which does not traverse the placenta, with flocculation techniques. This theory has been presented also in other publications (3, 4, 5), supported by a case (3) in which quantitative serological tests were made simultaneously from the blood of a syphilitic mother and her foetus. With

two different complement fixation tests the reagin titres were of the same size in maternal and foetal blood, with five different flocculation tests the level of reagin titres in the foetus was much lower than in the mother. Wiener (6) also published a case in which Kleine's flocculation test with the umbilical cord blood of the infant was much weaker than with the maternal blood but Kolmer's complement fixation test was only slightly weaker.

Nørgaard (7) collected 20 cases from Statens Serum Institut, Copenhagen, in which quantitative Wassermann and Kahn tests were made during delivery from the blood of a syphilitic mother and from the cord blood of the newborn child. The study showed that both the complement fixation and the flocculation antibody traverse the placenta though the titres in the cord blood are usually weaker than in the maternal blood. However, the difference between the maternal and the infantile Wassermann titre is on an average slightly smaller than the difference between the corresponding Kahn titres. There were 62 infants in Nørgaard's study who were at first seropositive but reverted to seronegativity without treatment and remained clinically asymptomatic during an observation period of at least one year. In 53 of these infants (85%) the umbilical cord blood was positive to the Wassermann test and in 43 (71%) it was positive to the Kahn test. Thus, with the complement fixation test, the frequency of positive cases was somewhat greater than with the flocculation technique, but the difference was not so marked as in Moore's series.

For checking Wiener's theory concerning transferable and non-transferable maternal syphilis reagins, we compared the blood specimens from syphilitic mothers treated at the Kumpula Hospital in the period 1949 to 1955 with those obtained from their newborn infants.

MATERIAL

The study is based on 211 seropositive mothers and their 213 infants, including two pairs of twins. In the case of the infants, all specimens were withdrawn from the umbilical cord. The specimens from the mothers were usually taken before delivery; the time of collection appears in table 2. At the same time quantitative serological tests (cholesterol Wassermann and Kahn) were made.

The specimens of cord blood were also studied by cholesterol Wassermann and Kahn tests, but unfortunately quantitative titration was not employed.

All the mothers had been treated with penicillin before delivery. The infants received no treatment but were followed at least until there was a reversal to seronegativity. The duration of observation up to the present time is shown in table 1.

TABLE 1

DURATION OF FOLLOW-UP OF INFANTS

Period of Observation	Test on Cord Blood Only	Less Than 6 Months	6 Months	1—2 Years	2—4 Years	Minimum 5 Years	Total
No. of infants	10	18	17	84	33	51	213

At the time of writing none of the infants has shown symptoms or signs of congenital syphilis.

RESULTS

Of the 213 infants in our series, 54 (25%) had a positive Wassermann test and 79 (37%) had a positive Kahn test. On the basis of these figures flocculating reagins seem to traverse the placenta even more readily than do the complement fixation reagins.

To compare the strength of the Wassermann and the Kahn reaction in the maternal blood, we computed the median of the respective titres (table 2). It was 3.2 dils for the Wassermann and 15.4 dils for the Kahn reaktion. This indicates that the Kahn test was distinctly more sensitive than the Wassermann test used. Table 2 shows, further, that a progressive, slight decrease in the medians

TABLE 2

TIME OF COLLECTION OF MATERNAL BLOOD SAMPLES, AND MEDIANS OF WASSERMANN AND KAHN TITRES

Withdrawal of Sample	Months				Total
	Before Delivery			After Delivery	
	6—4	3—2	1—0	0—1	
No. of mothers	54	55	78	24	211
Median of Wassermann dils.	3.5	3.3	3.2	2.2	3.2
Median of Kahn dils.	16.1	15.8	14.5	14.0	15.4

occurs during the last month of pregnancy and during the first month following delivery, and that the blood tests concerned are on an average indicative of the serological status of the mothers during 1 to 2 months before delivery.

TABLE 3

EFFECT OF TITRE OF MATERNAL WASSERMANN REACTION ON WASSERMANN-POSITIVITY OF THE INFANT

Maternal Wassermann Titre, Dils.	No. of Infants	Positive Wassermann in Cord Blood	
		No.	%
Negative	40	0	0
1— 2	93	22	24
4— 8	68	26	38
16—128	12	6	50
Total	213	54	25

TABLE 4

EFFECT OF TITRE OF MATERNAL KAHN REACTION ON KAHN-POSITIVITY OF THE INFANT

Maternal Kahn Titre, Dils	No. of Infants	Positive Kahn in Cord Blood	
		No.	%
1—2.5	30	4	13
5—10	109	29	27
20—150	74	46	62
Total	213	79	37

In tables 3 and 4 the results of the Wassermann and Kahn reactions of the infants are compared with the degree of positivity of the corresponding reactions in the mothers. They show that the number of infants giving a positive response increases steeply with the degree of positivity of the corresponding reaction in the maternal blood. It is also seen that all mothers had a positive Kahn test, whereas the Wassermann test was negative in 40 mothers. Thus the infants of these latter had not even a possibility of receiving complement fixing reagins from the blood of their mothers, and they were, in fact, all Wassermann negative.

When comparing the passing transferability of the complement fixing and the flocculating antibody through the placenta, it is

advisable to omit the 40 Wassermann negative mothers from the group concerned. The proportion of Wassermann positive infants then increases from 25% to 31%, and thus approaches the proportion of Kahn positive infants, 37%. This apparent predominance of the flocculating antibody disappears when account is taken of the fact that the Wassermann titre was high, over 8 dils, in the mothers of only 12 infants, but with the Kahn test the titre was high, over 10 dils, in the mothers of 74 infants, and that in the case of just these strongly seropositive mothers the frequency of both Wassermann and Kahn positive infants was highest. When comparing the groups of lower titres, it appears that in them the number of Wassermann positive infants was even greater than that of Kahn positive infants, but the difference is not statistically significant.

DISCUSSION

In the umbilical cord blood of the newborn infants of syphilitic mothers treated at the Kumpula Hospital, the Kahn test was more frequently positive than the Wassermann test. The result was thus contrary to the one that would be expected on the basis of Wiener's theory.

The maternal seroreactions showed that the Kahn test used in Finland is considerably more sensitive than the Wassermann test. When this is taken into consideration, the difference between the percentages of Wassermann and Kahn positive infants is reduced. Accordingly, both these reagins traverse the placenta about equally readily. Perhaps the differences in those studies that seem to support the Wiener theory are also due only to the varying sensitivity of the serological tests used.

Unfortunately, Moore's results cannot be checked with respect to the sensitivity of the serodiagnostic procedures: he gives no details as to the flocculation technique and the complement fixation test used. In addition, both tests were carried out on two different groups of infants.

Nørgaard's report, on the other hand, gives more detailed information. True, his series differs from ours in that it is based on cases from 1940—46, *i.e.* from the pre-penicillin period, whereas all mothers in the series from the Kumpula Hospital had been

treated with penicillin before delivery. Moreover, seronegative infants were only exceptionally accepted into the Danish file. Such a selection was not made in our material. These differences explain the fact that the proportion of seropositive infants in Nørgaard's series is higher than in ours.

A comparison of these two studies is facilitated by the fact that the sensitivity of the Kahn test is of the same degree in Denmark and Finland, for which we are indebted to the Venereal Disease Section of WHO.

The Wassermann test used in Denmark, however, is considerably more sensitive than our corresponding test and even more sensitive than the Kahn test, judging from the fact that, in Nørgaard's 20 syphilitic mothers, the average of the Wassermann titres was 5.8 and the average of the Kahn titres in the same mothers was 4.7. This difference probably accounts for the slight predominance of the positive Wassermann test over the positive Kahn reaction in the infants of Nørgaard's study.

Nørgaard's series also includes newborn babies with a stronger Kahn than Wassermann reaction. Such results show that the two isolated cases presented in support of Wiener's theory (3, 6) cannot be given much value when comparing the maternal and infantile seroreactions.

SUMMARY

Specimens of blood from 211 penicillin-treated mothers have been compared with specimens of cord blood from their 213 infants with the object of checking the theory presented by Wiener, according to which the complement fixing antibody passes through the placenta of a syphilitic mother more readily than the flocculating antibody. Of the infants, 25% showed a positive Wassermann test and 37% a positive Kahn test. The difference is reduced when taking into consideration that the Wassermann test employed was considerably less sensitive than the Kahn test. On the basis of the result here obtained both these antibody reagins traverse the placenta equally readily. In the studies performed elsewhere showing in the infants a predominance of the positive complement fixation test over the positive flocculation reaction, the former test has probably been more sensitive than the latter.

REFERENCES

1. Moore, J. E.: The Modern Treatment of Syphilis, ed. 3, Springfield, Charles C. Thomas, 1944, p. 486.
2. Wiener, A. S.: Ann. Allergy 1948:*6*:293.
3. Rein, C. R., and Konstant, G. H.: Arch. Derm. Syph. 1949:*60*: 217.
4. Rein, C. R.: Acta derm.-venereol. 1951, suppl. 24, p. 107.
5. Rein, C. R., and Reyn, Alice: Bull. Wld Hlth Org. 1956:*14*:193.
6. Wiener, A. S.: Ann. Allergy 1952:*10*:535.
7. Nørgaard, O.: Acta derm.-venereol. 1951:*31*:153.

THE EFFECT OF SOME POLYMERS ON INFLUENZA VIRUS

by

KARI PENTTINEN [1]

(Received for publication May 22, 1957)

In an earlier paper by the present writer the effect of poly-phloroglucinolphosphate on influenza virus was described. In some experimental conditions a depression of the infectivity and hem-agglutination titer of 3—4 logarithms was observed when eggs were treated with the preparation before injecting the virus. In vitro, however, the preparation also had a titer-decreasing effect and it was considered that the effect in vivo was possibly of the same pattern.

The present paper reports on some further experiments in which other polymers of the same type were tested for their antiviral activity. The experiments showed that two of them (polyphloretin-phosphate and compound PK 11 [2]) have the same type of effect both in vitro and in vivo although less in degree than poly-phloroglucinolphosphate.

Methods. — The egg experiments were carried out in the same way as those reported earlier (2). The in vitro inhibiting effect of the prepara-tions on the inactivation of virus hemagglutination inhibitor by active virus was studied. Virus strain Influenza A/Finland/1/51 was used in the egg and in the in vitro experiments. Diluted eggwhite was used as the virus hemagglutination inhibitor (3). In accordance with preliminary

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[2] »a high polymer substance which is negatively charged enzyme inhibitor with carbonic acid groups instead of phosphoric acid groups». The polymers were obtained by the courtesy of A. B. LEO, Hälsingborg and Dr. E. Dics-falusy to whose I am indebted.

REFERENCES

1. MOORE, J. E.: The Modern Treatment of Syphilis, ed. 3, Springfield, Charles C. Thomas, 1944, p. 486.
2. WIENER, A. S.: Ann. Allergy 1948:*6:*293.
3. REIN, C. R., and KONSTANT, G. H.: Arch. Derm. Syph. 1949:*60:* 217.
4. REIN, C. R.: Acta derm.-venereol. 1951, suppl. 24, p. 107.
5. REIN, C. R., and REYN, ALICE: Bull. Wld Hlth Org. 1956:*14:*193.
6. WIENER, A. S.: Ann. Allergy 1952:*10:*535.
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titrations the virus allantoic fluid was diluted in the test to 10^{-3} and egg-white to 10^{-2}. The dilution medium was bacteriological broth. The set-up of the experiments was as follows: To 0.4 ml inhibitor was added in 0.1 ml of bacteriological broth the polymer dilution in question, and to this 0.5 ml of virus allantoic fluid dilution. The tubes were kept in a water bath at $+37°$ C for 20 hours, after which the residual inhibitor content of the tubes was determined by heated (56° C, ½ hour) Influenza B virus strain Lee (3). The test tube contents were diluted in twofold steps in a 0.25 ml volume of saline in plastic plates, after which 0.25 ml of heated Lee allantoic fluid dilution containing four agglutinating units was added to each tube. After half an hour at room temperature, 0.5 ml of 0.5 per cent chicken cell suspension was added to each tube. The test was read after one hour at room temperature. Simultaneously, control tests were carried out in which, instead of polymer dilutions, 0.1 ml of broth was used to be sure of the activity of the virus and instead of the virus allantoic fluid dilution 0.5 ml of broth was used to be sure of the capacity of the inhibitor to inhibit the agglutination by heated Lee virus. When polyphloroglucinol-phosphate was in question the titrations were carried out in 4 per cent cholera vibrio filtrate treated chicken serum to prevent the agglutination caused by the preparation (2). The other preparations did not agglutinate chicken red cells.

RESULTS

Table 1 gives the results obtained with three different active polymers in tests where their capacity to inhibit the inactivation of eggwhite inhibitor by active virus was measured.

Table 1 shows that the effectiveness of polyphloroglucinol-phosphate by weight of preparation per ml is about one log higher

TABLE 1

TITERS OF RESIDUAL VIRUS HEMAGGLUTINATION INHIBITOR IN TWO EXPERI-
MENTS WERE THE CAPACITY OF THREE POLYMERS TO INHIBIT THE INACTIVATION
OF EGGWHITE INHIBITOR BY ACTIVE INFLUENSA VIRUS WAS MEASURED (SEE
UNDER »METHODS»). THE TITERS ARE EXPRESSED AS LOGARITHMS OF
RECIPROCALS OF DILUTIONS.

Dilution of Polymer	Exp. 1					Exp. 2				
	3	4	5	6		3	4	5	6	
Polymer										
Polyphloroglucinolphos-phate	1.5	1.2	1.2	0.6			1.8	1.8	1.2	
PK 11	1.2	1.5	0.6	0.6		1.8	1.8	1.5	0.6	
Polyphloretinphosphate	1.5	0.6	0.6	0.6		1.8	1.8	0.6	0.6	
Inhibitor control					1.5					2.1
Virus control					0.6					0.6

than that of compound PK 11 and about two logs higher than that of polyphloretinphosphate. Some other polymers have also been investigated, *e.g.* three different preparations of polyoestradiolphosphate (14, 17 and 113). All these were without effect in the same type of experiments.

All the six polymers mentioned above were also investigated in egg experiments. The results with three active polymers are presented in Table 2.

TABLE 2

THE EFFECT OF THREE POLYMERS IN DEPRESSING THE HEMAGGLUTININ PRODUCTION IN EMBRYONATED EGGS. THE POLYMERS WERE INJECTED INTO ALLANTOIC SACS 5 MINUTES BEFORE INJECTING THE EGGS WITH 10^3 I.D. 50 % OF INFLUENSA A VIRUS. THE INJECTED VOLUME OF THE POLYMER DILUTION WAS 0.5 ML. INCUBATION TIME AT $37°$ C, 24 HOURS. THE POLYMER DILUTIONS ARE EXPRESSED IN LOGARITHMS OF RECIPROCALS OF DILUTIONS. NOMINATOR = NUMBER OF EGGS SHOWING HEMAGGLUTININS. DENOMINATOR = NUMBER OF EGGS

Dilution of Polymer	2	3	4	5
Polymer				
Polyphloroglucinolphosphate ..		0/8	7/8	8/8
PK 11	2/7	6/6	7/7	
Polyphloretinphosphate	8/8	6/6		

Table 2 shows that of the three active polymers in the in vitro test the most effective was the one most effective in the egg experiment also, and the least effective had no effect in the egg experiment. Repeated tests gave the three polymers the same order of effectiveness. In a few experiments polyphloretinphosphate, too, had a very weak inhibiting effect on hemagglutinin production. All the other three polymers were without effect. Polyestradiol phosphate proved to be toxic for embryonated eggs.

DISCUSSION

The similarity of effect in the presented in vitro and in vivo experiments could be considered to favor the theory that the enzyme-like action of influenza virus with virus hemagglutination inhibitor is also necessary in an infectious process in eggs. However, the direct effect of polyphloroglucinolphosphate on influenza virus, which was shown in the earlier paper, is probably enough to explain the similarity. Distinction between the direct

effect of the polymer on virus and the effect of this polymer on the enzymelike action of virus on substrate is, however, difficult.

Taking into consideration the very slight, if any, avidity of polyphloroglucinolphosphate to produce precipitate with bacteriological broth and serum and its effect in the allantoic sac, it is not impossible that substances like these could have some effect also in animal experiments. In this connection, the size and the stability of the molecules are certainly of great importance.

REFERENCES

1. DICSFALUSY, E.: Endocrinology 1954:*54*:471.
2. PENTTINEN, K.: Ann. Med. exp. & Biol. Fenn. 1956:*34*:88.
3. PENTTINEN, K., and TOMMILA, V.: Ann. Med. exp. & Biol. Fenn. 1954: *32*:134.

FURTHER STUDIES ON THE GROWTH-CONTROLLING ACTION OF HUMAN SERUM ON HeLa CELLS[1]

by

KARI PENTTINEN and ERKKI SAXÉN

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In earlier papers (1. 2.) the present writers discussed the effect of human serum on cultures of HeLa cells. The individual variations were great and it was observed that, compared to cultivation in non-heated fresh human serum, the cultivation of HeLa cells in suitably inactivited serum caused higher cell counts and that the nuclear size in the latter was greater.

In our earlier papers we postulated the possible existence of a growth controlling system in the serum. Since elucidation of this question seemed to us to be of great potential value, the experiments were extended. In these experiments special attention was paid to the clumping of the HeLa cells occurring in nonheated sera. The present paper deals with this clumping phenomenon and the cytologic changes observed in connection with it. Interest was concentrated on the mode of growth of the cells in different heated and nonheated sera, but the enumeration of the nuclei after citric acid treatment was also carried out according to routine.

METHODS

Details of the technique used have been published in two earlier papers (1. 4.). In addition to the usual test tubes, cover glass tubes were also used and the cultivation of the HeLa cells was carried out

also in humidified 5 per cent CO_2 atmosphere in Petri dishes according to the technique presented by Puck et al. (3). The living cultures were observed after 20 and 44 hours growth under an ordinary microscope and the changes were recorded without any knowledge of the setup of the experiments. The cytologic changes were studied in stained slides and Petri dishes. The staining methods used were Feulgen nuclear reaction and haematoxylin eosin staining.

The inoculum size was about 25,000 cells per test tube.

Absorption experiments were carried out as described earlier (4). The sera used were collected in vacutainers (Behringwerke) the day before the experiment. The inactivation of the serum was carried out in a 55°C water bath during half an hour and the »nonheated sera» were placed in a 37°C water bath for the same time.

RESULTS

The Clumping Phenomenon. — When thirty per cent serum in Hanks' solution was used, the observation was made that between 10 and 20 per cent of the sera studied, when non heated, caused a clumping of HeLa cells and that this was not observed after inactivation of these sera. In most sera at thirty per cent serum level, as mentioned, no clumping was observed and accordingly the behavior of cells in nonheated and heated serum was very similar. The clumping phenomenon and the effect of inactivation is illustrated in Figs 1 and 2. The differences in nuclear sizes, as measured from stained smears after citric acid treatment, are shown in Fig. 3. It can be seen that in active serum the cells grow in dense compact colonies: the cells are smaller, the nuclei are generally rounded and the variation in nuclear size, as observed in stained smears, is small. Very little cytoplasm is visible around the nucleus and it often appears to have disintegrated. In inactivated serum the cells grow in a loose, highly extended meshwork and the surface area is thus much larger: the nuclei are large, round or oval and often irregular in shape. Some exceptionally large nuclei are also found. The nuclei are not as deeply stained with the Feulgen nuclear reaction as are those in nonheated serum and the nucleoli are prominent. The nuclear membrane seems to be thin, and the cells contain a large amount of cytoplasm, which is often vacuolated. The cell borders are distinct, the cells elongated and they have many
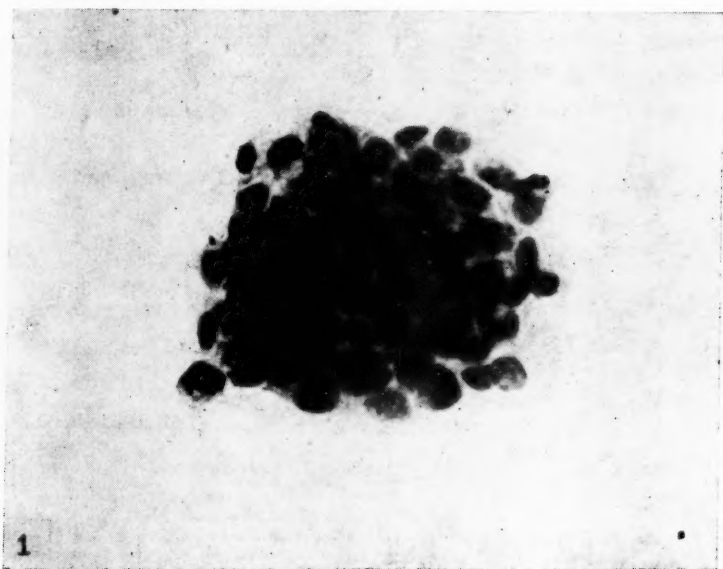
Fig. 1. — Photograph showing a dense compact colony of HeLa cells after two days growth in fresh active human serum. Feulgen nuclear reaction × 200.
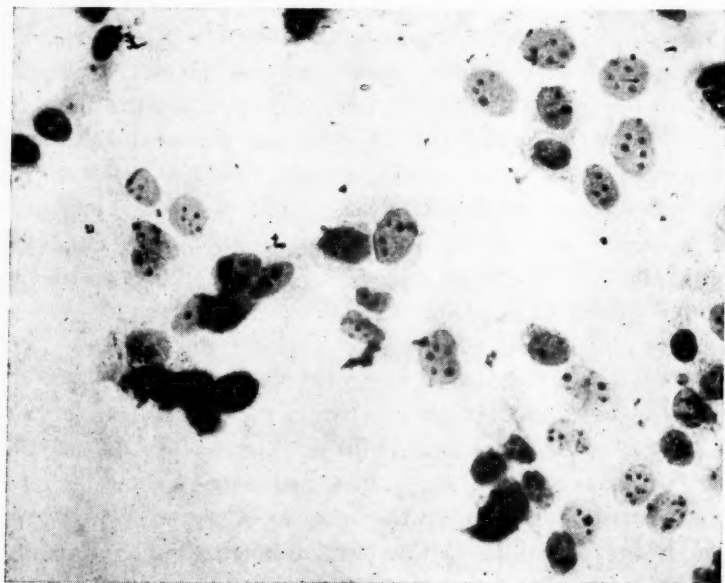
Fig. 2. — Photograph demonstrating the loose migratory structure of HeLa cell growth in inactivated human serum. (The same serum as in Fig. 1 after inactivation.) Feulgen nuclear reaction × 200.
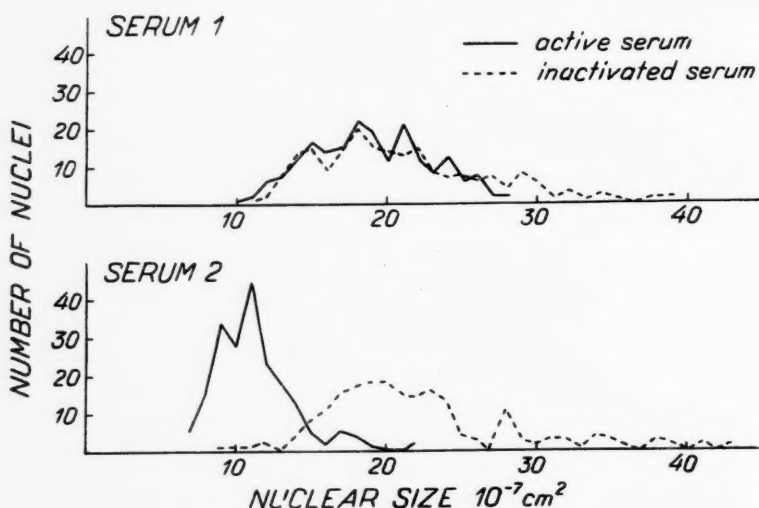
Fig. 3. — Distribution of nuclear sizes after two days growth in active serum and in inactivated serum. Serum 1 showed no clumping effect in 30 per cent concentration; in serum 2 the clumping effect was strong.

cytoplasmic processes with which they are connected with each other.

Effect of Serum Concentration. — All investigated sera, when non heated, caused the clumping phenomenon, if used concentrated. In those experiments where different serum concentrations were used, it was observed that if the clumping was weak in a 30 per cent serum, it was much stronger at the 60 and 90 per cent levels of the same serum. In no sera investigated was the clumping of the cells observed after inactivation of the serum — not even when used in the highest concentrations. The serum specimens collected repeatedly from the same individual behaved similarly.

Effect of Different Heating Temperatures and Storage. — The sera studied were incubated undiluted and tested at 30 per cent serum concentration. At an incubation time of half an hour for the sera in water baths of 37, 40, 45, 50, and 55°C, the clumping of the cells was observed only after incubation at the three lowest temperatures. From 50°C on the behavior of the cell was the same as after incubation at 55°C. After one week storage of serum in a + 4°C icebox, the clumping of the cells was very weak or not visible at all.

Effect of Absorption with HeLa Cells. — The success in improving the growth promoting capacity of active rabbit serum by absorption with HeLa cells (4) stimulated us to undertake similar experiments on the clumping phenomenon. As can be seen from Table 1 the treatment with HeLa cells removed or weakened the clumping factor(s) in the serum.

TABLE 1

THE DEGREE OF CLUMPING AND CELL COUNTS IN THOUSANDS AFTER TWO DAYS
GROWTH IN NONHEATED AND HEATED SERUM BEFORE AND AFTER ABSORPTION

Serum Concentration	Degree of Clumping and Cell Counts			
	Before Absorption		After Absorption	
	Active S.	Inactive S.	Active S.	Inactive S.
90 %	+ + (64)	— (83)	+? (105)	— (84)
30 %	+ (79)	— (69)	— (83)	— (77)

Inoculum size was 34,000 per test tube.

+ + = strong clumping   +? = weak clumping
+   = clear clumping   — = no clumping

Effect of Trypsin and Hyaluronidase. — When trypsin (''Difco,, 1:250) was present in nonheated ($\frac{1}{2}$ hour at 37°C) 50 per cent serum in a final dilution 1 : 2000 no clumping of cells was observed and the same result was obtained with hyaluronidase (100-200 TRU/mg ,,NBCo'') in a final dilution of 1 : 2000.

Testing of Individual Sera. — When laboratory personnel (22), aged persons (5), and untreated cancer patients (10) were investigated for the clumping phenomenon, no clear differences were observed. The number of clumping-causing sera at the 30 per cent level in these groups was 2, 2, and 1, respectively. The material is, however, too small to indicate any possible frequency differences.

DISCUSSION

The observed clumping phenomenon suggests that fresh, non heated, human serum contains a factor or factors which prevent the HeLa cancer cells from growing in a loose, migratory structure. Whereas individual variations are great, serum specimens collected from the same person at different times vary very little.

It is interesting to compare this observation with those pub-

lished by Puck et al. (3). They state that human sera contain a factor which causes the cells to grow in a loose highly extended meshwork, in contrast to some animal sera, in which the cells grow in dense compact colonies. It is not mentioned whether the sera used in these experiments were active or inactivated. The mode of growth obtained, however, may indicate, if the differences in serum concentrations are ignored, that inactivated serum was used; this seems to be the common practice in tissue culture work. At least the serum pools used were not fresh and, as observed in this investigation, sera stored for a long time in a + 4°C icebox have no clumping effect. Thus, the observations made by us and by Puck et al. are not contradictory. The differences between inactive (?) animal serum and inactive (?) human serum described in the paper cited seem to be of the same type as those presented here between fresh (active) human serum and inactivated human serum. This may indicate that the factors causing the clumping phenomenon in animal sera are not as termolabile as those in human serum. They might on the other hand also belong to a different group of factors, although the effect is very similar.

The absorption experiments show that the amount of clumping factor(s) in serum is limited and can be demonstrated only when the ratio of serum to cells is suitable. The inoculum size in experiments like these is thus, of primary importance.

The effect of trypsin and hyaluronidase is interesting and needs further study. The factors causing this clumping phenomenon may have some connection with the so called normal agglutinins in bacteriology.

The higher cell counts obtained in suitably inactivated sera, compared to counts in active serum, reported in a previous paper (2), were not observed in the present investigation. It is difficult to explain this failure after the initial success. It might be due to uncontrollable technical factors or to differences in the HeLa cell lines used. The original HeLa cell line was accidentally lost and the present experiments were carried out with a cell line stored in glycerol in a deep freezer (—20°C) for a long time.

The present paper clearly demonstrates the need of further studies with fresh individual human sera.

SUMMARY

The growth of HeLa cells was studied in fresh (active) and in inactivated individual human sera. The results tend to show that fresh active human serum contains a factor or factors which prevent the HeLa cancer cells from growing in a loose migratory structure. The cytologic changes observed are described and the effects of serum concentration, heating temperature, storage and absorption with HeLa cells are reported. The addition of trypsin and hyaluronidase seem to have a similar effect as inactivition on the mode growth. Sera from laboratory personnel, aged people and untreated cancer patients are compared.

REFERENCES

1. PENTTINEN, K., and SAXÉN, E.: Ann. Med. Exper. Fenn. 1956:*34*:22.
2. PENTTINEN, K., and SAXÉN, E.: Ann. Med. Exper. Fenn. 1956:*34*:242.
3. PUCK, T. T., MARCUS, P. I., and S. CIECIURA: J. Exp. Med. 1956:*103*:273.
4. SAXÉN, E., and PENTTINEN, K.: Ann. Med. Exper. Fenn. 1956:*34*:396.

ADENOVIRUSES ASSOCIATED WITH PHARYNGO-CONJUNCTIVAL FEVER [1]

ISOLATION OF ADENOVIRUS TYPE 7 AND SEROLOGICAL STUDIES SUGGESTING ITS ETIOLOGICAL RÔLE IN AN EPIDEMIC IN HELSINKI

by

N. OKER-BLOM, O. WAGER, HELENA STRANDSTRÖM, PIRJO MÄKELÄ, and ELLI JANSSON

In an earlier paper (4) a description was given of an epidemic associated with adenovirus infection. This epidemic outbreak occurred in Helsinki in the early fall of 1955. Most of the patients were children, and the swimming pools of the city seemed to play an important rôle in the spread of the epidemic. The clinical features of the disease resembled those of the pharyngoconjunctival fever described by Bell *et al.* (1), Berge *et al.* (2), Dascomb *et al.* (3), and Kjellén *et al.* (6).

From neutralization tests performed on some of the paired sera with the patients' own strain of virus and with the six proto-type viruses then available, it was concluded that the epidemic outbreak was caused by infection with adenovirus type 4 or a virus antigenically closely related to it.

In the present paper a more detailed report is given on the agents isolated in connection with this epidemic. Antibody determinations also have been carried out by means of neutral-ization and complement fixation tests. In addition to paired sera from patients involved in the epidemic, also other sera from patients

treated at the Aurora Hospital during 1955—56 were examined for antibodies against adenoviruses. This was done with the purpose of obtaining some general information of the immunity status in Helsinki against adenoviruses.

METHODS

HeLa Cell Cultures. — The cells were maintained in one liter Roux flasks by a technique described elsewhere (7). This technique, however, was modified as follows. To the nutrient fluid for primary cultivation was added tryptose phosphate broth (Bacto) 0.2 per cent, found by Ginsberg *et al.* (5) to stimulate the multiplication of the HeLa cells. Each Roux flask was inoculated with 80 in stead of 70 ml of a suspension of 50,000 in stead of 80,000 to 100,000 cells per ml of medium.

The HeLa cell tube cultures to be used for neutralization and virus isolation experiments also were prepared as described earlier (7).

Technique for Virus Isolation. — The stool specimens were prepared according to the method described elsewhere (7).

For throat washing ordinary broth was employed. To the washings 100 IU of penicillin and 100 μg of streptomycin per ml were added. Centrifugation at 15,000 r.p.m. at 5°C for 15 minutes followed. The supernatant was used for inoculation in HeLa cell tubes.

For isolation of virus each specimen was inoculated into three tubes. In the case of throat washings, the inocula were 0.2 ml. In the case of stool specimens, inocula of 0.1 ml mostly were used. For second and further passages inocula of 0.1 ml always were used. The tubes were inspected daily for cytopathogenic effects up to a maximum of 10 days.

Prototype Virus Strains. — Strains of Types 1—6 were obtained through the courtesy of Dr. Arne Svedmyr, Stockholm, and strains of Types 7—11 through the courtesy of Dr. C. H. Andrews, London.

For virus propagation, Carrell flasks with a diameter of 60 mm and inoculated with 8 ml of HeLa cells were employed. Complete disintegration of the HeLa cells was usually seen 4—5 days following inoculation with one ml of virus suspension. The virus suspension was stored in a deep-freeze at —10°C to —18°C until investigated. This virus suspension, after centrifugation at 1,500 r.p.m. for 10 minutes, was used as antigen for production of hyperimmune sera in rabbits.

Production of Hyperimmune Sera. — Rabbits were injected intravenously every second day with one ml of virus suspension. Six injections were given to each animal. One month later the rabbits were boosted with a seventh similar injection of virus suspension. One week following this injection the rabbits were bled either by heart puncture or from the carotid artery. The serum was separated and inactivated, then stored in a deep-freeze until used. The neutralizing titers of the immune sera thus obtained ranged from 1 : 400 to 1 : 800.

Titration of Virus Strains. — A dilution series with ten-fold dilutions of the virus suspension was prepared using as diluent Parker 199 mixture. 0.1 ml of each dilution was inoculated to four HeLa cell tubes, and the degree of cell destruction investigated after an incubation of 5 days. The CPD_{50} of the virus was calculated according to the method of Reed and Muench (8). The CPD_{50} titers of the different virus strains tested varied from 10^{-2} to 10^{-4}.

Neutralization Test. — All the sera were inactivated by incubating them at 56°C in a water-bath for 30 minutes. To 0.25 ml of a serum dilution of 1 : 4 an equal volume of virus suspension containing 100 CPD_{50} per 0.1 ml was added. The serum virus mixture was allowed to stand in room temperature for one hour. Then 0.2 ml of the mixture was inoculated into two HeLa cell tubes. The results were examined after 5 days.

For typing of virus strains isolated from patients, 0.25 ml of a dilution of rabbit immune serum, varying from 1 : 10 to 1 : 30, and an equal volume of the undiluted virus suspension were mixed. The HeLa cell tubes inoculated with 0.2 ml of this mixture were examined daily. In a number of instances it was necessary to titrate the virus and then re-examine the neutralizing capacity of the immune sera using 100 CPD_{50} of the virus in the test.

Complemet Fixation Test. — The specific antigen was prepared as follows. One liter Roux flasks, inoculated with 80 ml of HeLa cell suspension and incubated for 4—5 days, were washed with Hanks solution. The fluid was then replaced by Parker 199 mixture, containing 5 per cent of inactivated chicken serum and the usual amounts of antibiotics. Inoculation with one ml of adenovirus type 2 suspension (F—1526) followed. After an incubation period of 5—6 days the suspension was harvested and titrated in HeLa cell tubes. The titer was commonly around 10^{-3}.

The non-specific antigen was prepared as follows. Carrell flasks with a diameter of 60 mm, inoculated with 8 ml of HeLa cell suspension and incubated for 4—5 days, were treated as described above, omitting, however, the inoculation with adenovirus. After an incubation period of 5—6 days the fluid medium was harvested. The HeLa cells, adhering to the walls of the flask, were liberated by treatment with trypsin as described elsewhere (7). Following two washings with Parker 199 mixture the cells were resuspended in the fluid medium.

Both the specific, virus containing antigen and the nonspecific antigen were then frozen by agitating the tubes in a mixture of alcohol and dry ice, and again thown in a water-bath at 37°C. This procedure was repeated tentimes. After centrifugation at 1,500 r.p.m. for 15 minutes the supernatants were collected and stored in a deep-freeze until used as antigens in complement fixation tests.

In the performance of the complement fixation test the procedures recommended by WHO (10) were followed with certain modifications. Due to the relatively low titers of the hemolysins available, the dilutions used in the titration of the hemolysin were 1 : 1,000 — 1 : 2,000 — 1 : 3,000

— 1 : 4,000 — 1 : 5,000 — 1 : 6,000 — 1 : 8,000 — 1 : 10,000 — 1 : 12,000.
A 2.5 per cent in stead of 2 per cent suspension of sheep cells was used
throughout the tests. In the titration of the complement, a final dilution
of 1 : 15 proved suitable.

RESULTS

In all, throat washings and/or stools from 27 out of the total
of 57 children treated for pharyngoconjunctival fever at the
Aurora Hospital were investigated for the presence of virus in
tissue culture. From several of the patients acute phase and con-
valescent sera were tested for the presence of complement fixing
and/or neutralizing antibodies against adenoviruses.

Table 1 shows the data obtained in these investigations.

From the data of table 1 it can be seen that adenovirus type 7
was recovered from 24 out of the total of 27 patients. Adenovirus
type 3 alone was recovered from one patient, and from two patients
both adenovirus type 3 and 7 were recovered. Thus, in 25 cases
out of a total of 27 adenoviruses could be isolated either from
the stool or the throat or both, and only two cases were negative
in this respect. Both these patients were secretors of poliovirus
type 2. In all, polioviruses were isolated from five patients (type 1
twice, type 2 three times).

Paired sera from 12 patients were examined for the presence
of neutralizing antibodies against adenovirus type 7. In all but
one instance either an appearance of antibodies in the second
bleeding or a fourfold or greater increase in the titer was seen.
In 11 instances paired sera were titrated for complement fixing
antibodies against adenoviruses. A definite increase in the titer
was seen in five cases only. Considering the known pattern of an
earlier disappearance of complement fixing antibodies as compared
to that of neutralizing antibodies, the interval between the first
and the second bleeding may have been much too long in four
instances (cases 3, 4, 5, 12). In accordance with this pattern are
our data in cases 2. and 5., in which there was seen a definite
drop in the complement fixing titer 19 months following the illness,
at a time when neutralizing antibodies still were present in the
circulation.

Table 2 elucidates the immunity status of five patients in
April 1957 as compared to that in September 1955, when they

TABLE 1

DATA OF VIRUS ISOLATION AND ANTIBODY ASSAYS ON 27 CHILDREN TREATED AT THE AURORA HOSPITAL FOR EPIDEMIC PHARYNGOCONJUNCTIVAL FEVER IN THE FALL OF 1955

Case No.	Journal No.	Age Yrs.	Virus Isolated from		Antibodies against Adenoviruses			
			Stool [2]	Throat [2]	Date	Days after Onset of the Illness	Complement Fixing [1]	Neutralizing against Adeno 7
1	2573	8	Adeno 3 (14)	Adeno 7 (12)	9. 9. 55	12	4	4
					9. 20. 55	23	16	256
					4. 9. 57	585	2	32
2	2525	11	» 7 (7)	» 7 (6)	9. 5. 55	6	4	—
					9. 16. 55	17	256	64
					4. 10. 57	585	16	+
3	2510	7	» 7 (4)	» 7 (4)	9. 5. 55	5	4	—
					4. 9. 57	580	16	4
4	2671	13	Polio 1 (4)	» 7 (4)	9. 17. 55	4	4	—
					4. 10. 57	570	16	32
5	2456	9	Adeno 7 (11)		9. 5. 55	14	64	—
					4. 10. 57	590	16	32
6	2469	10	» 7 (3)	Polio 2 (2)	9. 5. 55	7		64
					9. 15. 55	17		256
7	2486	9	» 7 (6)	Adeno 7 (5)	9. 5. 55	8	4	+
					9. 16. 55	19	16	+
8	2526	11	» 7 (6)	— (6)	9. 6. 55	5		—
					9. 14. 55	14		256
9	2545	10	» 7 (4)	Adeno 7 (3)	9. 6. 55	3		—
					9. 17. 55	14		256
10	2585	8	» 7 (20)	— (19)	9. 11. 55	19		—
					9. 22. 55	30		+
11	2574	12	» 7 (15)	— (11)	9. 9. 55	11	256	256
					9. 22. 55	24	512	1024
12	2623	7	» 7 (16)	Adeno 7 (13)	9. 14. 55	14	4	—
					2. 7. 56	145	4	320
13	2544	13	— (4)	» 7 (4)	9. 6. 55	4	4	
					9. 17. 55	15	8	
14	2638	7	Adeno 7 (6)	» 7 (6)	9. 16. 55	7	4	
15	2661	3	» 7 (7)	» 7 (6)	9. 16. 55	6	4	
16	2468	9	» 7 (10)	» 7 (10)				
17	2465	11	» 7 (4)	» 7 (3)				
18	2481	6	» 7 (7)	» 7 (6)				
19	2475	10	» 7 (4)	— (4)				
20	2493	12	» 7 (11)	— (10)				
21	2624	7	» 7 (9)	Polio 1 (10)				
22	2702	1	» 7 (8)					
23	2739	8	» 7 (4)					
24	2464	10	» 3 (8)	— (6)				
25	2570	12	» 3 (5)	Adeno 7 (5)	9. 8. 55	5	4	
26	2568	11	Polio 2 (1)	— (2)				
27	2557	9	» (4)	Polio 2 (1)	9. 8. 55	2	4	
					9. 19. 55	13	4	

+ = Neutralization in serum dilution 1 : 4, titration not done.
— = No neutralization in serum dilution 1 : 4.
[1] As antigen adenovirus type 2.
[2] The number in brackers designates time in days from the onset of the illness to the time when the specimens were taken.

TABLE 2

NEUTRALIZING ANTIBODIES AGAINST ADENOVIRUSES IN THE SERA TAKEN FROM
FIVE CHILDREN IN THE ACUTE PHASE OF PHARYNGOCONJUNCTIVAL FEVER AND
IN THEIR SERA TAKEN NINETEEN MONTHS LATER

Adenovirus	V. I. (2525)		L. B. (2573)		E. P. (2671)		J. T. (2456)		P. K. (2510)	
	I Sept. 1955	II Apr. 1957	I Sept. 1955	II Apr. 1957	I Sept. 1955	II Apr. 1957	I Sept. 1955	II Apr. 1957	I Sept. 1955	II Apr. 1957
Type 1	—	—	—	—	—	—	+	+	+	+
Type 2	·	+	—	—	+	+	+	+	+	+
Type 3	—	—	+	+	—	—	—	—	—	—
Type 4	±	—	±	—	+	+	+	+	—	+1 : 32
Type 5	—	—	—	+	—	—	—	—	—	—
Type 6	—	—	—		—	—	—	+	—	—
Type 7	—	+	—	+1 : 32	—	+1 : 32	—	+1 : 32	—	+1 : 4
Type 8	+	+	+	+	+	+	+	+	+	+
Type 9	+	+	—	—	—	—	—	—	—	—
Type 10	+	+	—	—	+ ·	+	+	+	—	—
Type 11	+	+	+	+	+	+	+	+	+	+
Titer of Complement Fixing Antibodies (Antigen Type 2)	1 : 4	1 : 16	1 : 4	1 : 16	1 : 4	1 : 16	1 : 64	1 : 16	1 : 4	1 : 16
Virus Isolated from Stool	Adeno Type 7		Adeno Type 3		Polio Type 1		Adeno Type 7		Adeno Type 7	
Throat	Adeno Type 7		Adeno Type 7		Adeno Type 7		—		Adeno Type 7	

+ = Neutralization in serum dilution 1 : 4, titration not done.
— = No neutralization in serum dilution 1 : 4.

were treated at the hospital and the acute phase sera were collected.

From the data of table 2 it can be seen that none of the patients had neutralizing antibodies against adenovirus type 7 in the acute phase of the pharyngoconjunctival fever in September 1955, whereas all five of them had neutralizing antibodies in their sera 19 months later against the same virus. Except for this consistent change in the immunity status against adenovirus type 7, remarkably few changes had taken place in the immunity status against other types of adenoviruses during this period of time. Antibodies

against type 6 had appeared in one instance (J. T.), against type 5 in one instance (L. B.), and against type 4 in one instance (P. K.). This latter phenomenon may possibly be due to neutralizing antibodies against type 7, cross reacting with the antigenically related type 4 virus, as shown by others (9).

Furthermore, it can be seen from table 2 that neutralizing antibodies against all 11 types of adenoviruses tested were present in the sera of one or more of the five patients. Most commonly found were neutralizing antibodies against adenoviruses of types 8 and 11, which were present in all sera tested. Only one patient had antibodies against adenovirus type 3 (L. B.) in her serum, and at the same time she was the only patient, from whom adenovirus type 3 could be recovered.

In order to obtain more general information on the immunity status in Helsinki against adenoviruses, 190 paired sera were titrated for the presence of complement fixing antibodies against adenoviruses. These sera had been sent to the laboratory from the wards of the Aurora Hospital in 1955—56, most of them for antibody determinations against polioviruses. Some of the sera showing high titer of complement fixing antibodies were then tested for neutralizing antibodies against adenoviruses of types 1—11.

In table 3 are shown the data obtained in the complement fixation tests.

From the data in table 3 it can be seen that a four-fold or greater increase in the complement fixing titer in the convalescent serum as compared to the acute phase serum was observed in 10 cases only, out of a total of 190 paired sera tested. The peak

TABLE

DISTRIBUTION OF THE TITERS OF COMPLEMENT FIXING ANTIBODIES AGAINST
AURORA HOSPITAL FOR VARIOUS

Blood Specimen Sent to the Laboratory for Antibody Assay against	Acute Phase Serum						
	$\leqq 4$	8	16	32	64	128	256
Polioviruses (1955)	27	8	16	4	4	1	—
Polioviruses (1956)	27	3	19	6	6	2	—
Other viruses (1956)	40	7	16	2	2	—	—
Total	94	18	51	12	12	3	—

of the titer in all the 10 cases remained below 1 : 32. In the paired sera of 28 patients a titer of 1 : 32 or more was encountered. None of these patients showed a four-fold or greater increase of the titer between the acute phase and the convalescent serum.

The clinical diagnoses of the 28 patients, whose sera showed a titer of 1 : 32 or more, were the following. Paralytic poliomyelitis in 11 cases, serous meningitis in 10 cases, paresis of the facial nerve in 2 cases, encephalitis in one case, epidemic parotitis in one case, infectio acuta in one case, maxillary sinusitis in one case, and disseminated sclerosis in one case.

Paired sera from 11 patients with a complement fixing titer of 1 : 32 or more were then tested for neutralizing antibodies against adenoviruses of types 1—11.

Neutralizing antibodies aga'nst all 11 types of adenoviruses tested were frequently found among the paired sera from these 11 patients.

DISCUSSION

The data presented give evidence for the view that the epidemic of pharyngoconjunctival fever which occurred in Helsinki in the early fall of 1955 was caused by adenovirus type 7. This agent was recovered from 24 out of a total of 27 children examined. The serological data, especially the rises in neutralizing and/or complement fixing antibodies in 12 instances out of a total of 15 paired sera examined, indicate that the infections with type 7 virus occurred simultaneously with the acute symptoms of disease.

On the basis of a few neutralization tests, described in an earlier

3

ADENOVIRUSES AMONG 190 PAIRED SERA FROM PATIENTS TREATED AT THE DISEASES IN 1955—56

Convalescent Serum							Total Number of	
⩾4	8	16	32	64	128	256	Paired Sera Tested	Paired Sera with a Fourfold or Greater Increase in the Titer
29	5	19	2	4	1	—	60	7
28	4	14	10	4	3	—	63	2
40	8	15	1	2	—	1	67	1
97	17	48	13	10	4	1	190	10

paper (4), it was concluded that the isolated viruses were identical or at least antigenically closely related to the type 4 prototype strain. For these neutralization tests, only prototype strains 1—6 were available. Therefore, a more accurate analysis could not be accomplished, in order to exclude the possibility of cross neutralization observed by others (9) to occur between types 4 and 7.

Neutralization tests carried out with the sera of patients treated at the Aurora Hospital in 1955—56 for various diseases suggest that infections caused by adenoviruses of types 1—11 are not infrequent in Helsinki. Neutralizing antibodies against all eleven types of adenoviruses were encountered in the sera examined. Due to the small size of the series and to the selection involved in collecting the samples, no conclusions are justified as to the quantitative aspects on the occurrence of the various types of adenoviruses.

SUMMARY

Data are presented suggesting that adenovirus of type 7 was the causative agent of an epidemic outbreak of pharyngoconjunctival fever which occurred in Helsinki in the early fall of 1955. Adenovirus type 7 was recovered from 24 out of a total of 27 children examined. A rise in neutralizing and/or complement fixing antibodies was seen in 12 instances out of a total of 15 paired sera examined.

Complement fixation tests were performed on 190 paired sera derived from patients treated for various diseases at the Aurora Hospital in 1955—56. Eleven paired sera with high titer of complement fixing antibodies were tested by neutralization tests against adenoviruses of types 1—11. Neutralizing antibodies were encountered against all eleven types among the sera studied. Thus, infections caused by adenoviruses of types 1—11 apparently are not infrequent in Helsinki.

Note. — After the completion of this paper Rowe has reported that one of the adenovirus strains isolated by us (see table 1, Journal No. 2545) and sent to him for typing proved to belong to subgroup 7 a.

REFERENCES

1. BELL, J. A., ROWE, W. P., ENGLER, J. I., PARROTT, R. H., and HUEB-
   NER, R. J.: J. A. M. A. 1955:*157:*1083.
2. BERGE, T. O., ENGLAND, B., MAURIS, C., SHUEY, H. E., and LENNETTE
   E. H.: Am. J. Hyg. 1955:*62:*283.
3. DASCOMB, H. E., and HILLEMAN, M. R.: Am. J. Med. 1956:*21:*161.
4. FORSSELL, P., LAPINLEIMU, K., STRANDSTRÖM, H., and OKER-BLOM,
   N.: Ann. med. exper. et biol. Fenniae 1956:*34:*287.
5. GINSBERG, H. S., GOLD, E., and JORDAN, W. S.: Proc. Soc. Exper.
   Biol. & Med. 1955:*89:*66.
6. KJELLÉN, L., STERNER, G., and SVEDMYR, A.: Acta Paediatrica 1957:
   *46:*164.
7. OKER-BLOM, N., and STRANDSTRÖM, H.: Ann. med. exper. et biol.,
   Fenniae 1956:*34:*177.
8. REED, L. J., and MUENCH, H.: Am. J. Hyg. 1938:*27:*493.
9. ROWE, W. P., HARTLEY, J. W., and HUEBNER, R. J.: Proc. Soc.
   Exper. Biol. & Med. 1956:*91:*260.
10. WHO Seminar on the Public Health Laboratory Aspects of Virus
    and Rickettsial Diseases, Madrid 1956.

C REACTIVE PROTEIN IN SEROUS MENINGITIS AND PARALYTIC POLIOMYELITIS

by

ODD WAGER [1] and ELLI JANSSON

C Reactive Protein (CRP) is a pathological protein occurring in human serum in various conditions connected with inflammation of infectious or non-infectious origin. This protein is characterized by its ability to form precipitates with the somatic pneumococcal C polysaccharide (9). Taking advantage of the antigenic specificity of the CRP, differing from other human serum constituents, specific antisera can be produced by immunizing rabbits with purified CRP (6, 7). Using such antisera, small amounts of CRP may be detected in human serum.

In recent years, the determination of CRP has gained clinical significance as a non-specific but sensitive test for estimation of the activity of the disease process in rheumatic fever (1, 2, 4, 8), myocardial infarction (5, 10), and infectious hepatitis (3).

The studies to be described in the present paper were carried out with the purpose of gaining information on the possible occurrence of CRP in connection with serous meningitis and paralytic poliomyelitis. Such an investigation seemed to us particularly suggestive considering the insignificant or no elevation of the sedimentation rate (SR) occurring during the course of these diseases. Thus, it was hoped that the CRP test might offer a means of value for estimation of the activity of the disease process.

METHODS AND RESULTS

The CRP Test. — A commercial rabbit immune serum, capillary tubes, and a special rack, all delivered by Schieffelin & Co., New York, were used. The tests were carried out as follows:

1.5 cm each of CRP antiserum and patients' serum were drawn up into the capillary tube and incubated for two hours at $+ 37°$ C. The tubes were kept in the refrigerator overnight, the degree of precipitate formed was then read against a dark background. The readings were recorded in millimeters of precipitate formed.

In all, 22 patients were included to this study. Out of them, 10 were treated for paralytic poliomyelitis, 12 for serous meningitis. The causative agent of serous meningitis in five cases apparently was mumps virus, in most or all remaining cases probably poliovirus type 3. In the fall of 1956, an epidemic caused by poliovirus of this type was going on in Helsinki. All the patients were admitted to the hospital between October 1956 and January 1957, 19 of them in October or November of 1956. The age of the patients ranged from two years to 44 years, seven of them being less than 15 years of age. 12 of the patients were female, 10 male.

In table 1 are shown data from these studies.

From the data of table 1 it occurs that practically no measurable CRP occurred in the circulation of the 22 patients studied. In all, 74 serum samples taken at various stages of the illness were tested for the presence of CRP. In 68 sera no CRP whatsoever was detectable, three sera gave a $\pm$ reading, whereas only three sera gave a definitely positive reading. Out of the three latter sera, two were post tracheostomy sera taken from a patient with acute progressive respiratory paralysis. Even in this case (No. 8), two serum specimens taken previous to the tracheostomy gave negative results in the CRP test, in spite of a relatively high simultaneous sedimentation rate (SR 43 and 38 mm/hour). Thus, in the case of this patient the two positive CRP readings probably were due to the postoperative inflammatory reaction caused by the tracheostomy. There remained only one single serum specimen (Case No. 22) with a definitely although weakly positive CRP reading, possibly caused by the disease process of serous meningitis.

The results obtained in the CRP and SR tests are summarized in table 2.

TABLE 1

CRP TESTS AND SEDIMENTATION RATES AT SERIAL BLEEDINGS OF 10 PATIENTS WITH PARALYTIC POLIOMYELITIS AND 12 PATIENTS WITH SEROUS MENINGITIS

Case No.	Dg.		Days after Admission to the Hospital															Virus Isolated	Antibodies in Acute Phase and Convalescent Sera against		No. of Cells in Cerebrospinal Fluid
			2	4	6	8	10	12	14	16	18	20	22	24	26	28	30		Polio 3 (Neutr.)	Mumps (CF)	
1		CRP		−	−		−			−								−	ECHO?	16/16		136
		SR		23	18		14			11								8				
2		CRP	−		−	−							−					−	Polio 3	64/64		87
		SR	10		9	8							9					5				
3		CRP				−	−		−			−							None	64/64		218
		SR				20	18		11			7										
4	Paralytic Poliomyelitis	CRP	−		−	−	−												Polio 3			296
		SR	5		8	15	10															
5		CRP					−												None			2?
		SR				20	14			11												
6		CRP								−	−								None	16/64		25?
		SR	28							9	6											
7		CRP			−	−	−		−										Polio 3	256/256		3?
		SR			15	23	7		6													
8		CRP	−		−	4+			3+				−						None	16/64		50?
		SR	43		38	43			79				36									
9		CRP			−		−			−									Polio 3	/1024		296
		SR			7		8			7												
10		CRP	−	−	−				−										Polio 3	4/16		38?
		SR	10	7	11				3													
11		CRP						−													64/64		136
		SR	11					10	11													
12		CRP					−			±			±								<4/<4		8?
		SR				11	28			18			13										
13		CRP	−		−		−		−										None	4/4		119?
		SR	30		20	22	13		10													
14		CRP							−				−						None	<4/<4		330
		SR					5		4				3										
15	Serous Meningitis	CRP						−		−									None	<4/<4		146
		SR				32	30		20													
16		CRP								−	−								None	64/64		40
		SR	20								4											
17		CRP	−	−	−															<4/<4	8/64	107
		SR	13	10	5				5													
18		CRP	−	−	−														None		64/128	108
		SR	27	28	16				6													
19		CRP					−	−	−	±										4/4	8/16	600
		SR					11	11	8	9												
20		CRP				−															16/256	215
		SR			18	12			8													
21		CRP	−		−	−		−											Polio 3	<4/<4		87?
		SR	18		15	17		5														
22		CRP				−	1+		−			−							None	<4/<4	16/256	60?
		SR				15	21		26			18										

Note. — In cases No. 17—20 and 22 epidemic parotitis preceded the onset of serous meningitis.

TABLE 2

CRP AND SR IN SEROUS MENINGITIS AND PARALYTIC POLIOMYELITIS

CRP		SR		
		Number of Sera		
+	—	>15 mm/hr	10—15 mm/hr	<10 mm/hr
3 (2)	71 (22)	30 (14)	26 (15)	28 (15)
Per cent 4	96	36	31	33

The number of patients from which the sera derived is given in brackets.

DISCUSSION

The studies described justify the conclusion that the inflammatory reaction associated with serous meningitis and paralytic poliomyelitis does not generally cause any appearance of CRP in the circulation. Out of 74 patient sera examined only three contained amounts of CRP, measurable by the antiserum test. Out of these, the appearance of CRP in two serum specimens from one patient doubtlessly was caused by postoperative inflammatory reaction, and not by the disease process in the central nervous system.

Most of the patients studied were severely ill, with high fever and in most instances with more than 100 cells per ml of cerebrospinal fluid. Therefore, it was somewhat surprising to find out that no CRP appeared in their circulation during the acute phase of the illness. This finding is in striking contrast to the behaviour of the CRP test as an extraordinary sensitive indicator of inflammation associated with rheumatic fever (1, 4, 8), hepatitis (3), and myocardial infarction (5, 10), equal with or even supreme to the sedimentation rate. In our series of 22 patients, the CRP test proved definitely less sensitive than the SR as an indicator of inflammation.

The etiology of the disease of most or all of the 22 patients studied was a virus infection. It remains to be studied, whether the viral etiology or the location of the disease process to the central nervous system is the characteristic feature of an inflammation not-connected with an appearance of CRP in the circulation.

The differential diagnosis between serous and purulent meningitis may sometime be difficult in the earliest phase of the illness. A study on the possible serviceability of the CRP test as a complementary aid in the early differential diagnosis between these clinical entities is under progress.

SUMMARY

Twenty-two patients were investigated by repeated bleedings for the occurrence of C Reactive Protein (CRP) during the course of serous meningitis and paralytic poliomyelitis. Practically no occurrence of CRP was encountered in 74 serum specimens taken from these patients, whereas a sedimentation rate of more than 15 mm/hour was seen in 36% of the sera examined.

The results are discussed.

REFERENCES

1. ANDERSON, H. C., and McCARTY, M.: Amer. J. Med. 1950:8:445.
2. HEDLUND, P.: Acta med. Scandinav. 1947:Suppl.196:588.
3. HEDLUND, P.: Nord. med. 1956:56:1505.
4. KROOP, I. G., HEFFER, E. T., and SHACKMAN, N. H.: Clin. Res. Proc. 1954:2:39.
5. KROOP, I. G., and SHACKMAN, N. H.: Proc. Soc. Exper. Biol. & Med. 1954:86:95.
6. MACLEOD, C. M., and AVERY, O. T.: J. Exper. Med. 1941:73:183.
7. MACLEOD, C. M., and AVERY, O. T.: J. Exper. Med. 1941:73:191.
8. STOLLERMAN, G. H., GLICK, S., and PATEL, D. J.: Am. J. Med. 1953: 15:645.
9. TILLETT, W. S., and FRANCIS, Jr., T.: J. Exper. Med. 1930:52:561.
10. WAGER, O., and HÄLLSTRÖM, K.: Cardiologia 1956:29:321.

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POSTNATAL ANTITOXIN RESPONSE OF THE CHICKEN

EFFECT OF FETAL CONTACT WITH DIPHTHERIA TOXOID

by

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The theory of antibody production proposed by Burnet and Fenner (5) implies that foreign antigens introduced into an animal during the prenatal period of its life will thereafter be treated as autogenous, so that later exposure provokes no immunological response to the same antigen.

Stimulated by this postulation, a number of authorities in recent years have attempted to provide an experimental basis for this concept. A variety of antigens and experimental conditions have been chosen. Results supporting the hypothesis of the existence of such an inhibitory effect on the immune response by perinatal contact with an antigen have been reported by some investigators (4, 7, 8, 10, 12), whereas others have not been able to observe such an effect (1, 2, 6).

In many of the studies reported rather rough and inaccurate methods have been used for evaluation of the immune responses observed. In none of them has the diphtheria toxin antitoxin system been employed as an immunological tool. Considering the extraordinary degree of sensitivity and specificity of the rabbit skin method for evaluation of the antitoxin response, it seemed to us well-founded to attack this problem by the use of this single antigen antibody system as immunological tool. As experimental animal

the chicken was chosen, primarily because of the possibility thus obtained of introducing the antigen to the embryos by the intravenous route.

Diphtheria Toxoid. — The toxoid preparation used in the experiments was made by Lääketehdas Orion, Medical Manufacturers, Helsinki, and was obtained through the courtesy of dr. Lauri Jännes, Ph.D. This preparation contained 1700 Lf of specifically precipitable toxoid per mg of protein nitrogen.

The Freund adjuvant containing heat-killed tubercle bacilli was prepared following the directions given by Cohn (9). A Waring Blendor was used for mixing the ingredients of the toxoid adjuvant mixture.

Assay of Diphtheria Antitoxin. — The technique of the intracutaneous rabbit test was followed (11) with the exception that, for reasons of economy, guinea pigs were used instead of rabbits. The toxin preparation, the standard antitoxin, and additional details on the assay technique are described elsewhere (13).

Intravenous Injection of the Chicken Embryos. — The technique of exposing the vein was that described by Beveridge and Burnet (3). The shell membrane was made transparent by a drop of sterile light paraffin oil (Bayol F, Stanco Distributors, New York). For injection of the test fluid, a 0.25 ml syringe and a 27 gauge needle were employed. The window was sealed with Scotch tape.

Series I. — This series consisted of 93 fertilized eggs. On the 12th day of incubation, 49 eggs were injected by the intravenous route with 34 Lf of fluid toxoid, contained in 0.1 ml of diluent. The remaining 44 eggs served as controls, and were similarly injected with 0.1 ml of diluent alone.

After the injections, incubation was continued until hatching, which was reached in 13 cases in the toxoid group and in 12 cases in the control group. At various intervals following the hatching a challenging dose of 34 Lf of fluid toxoid was given subcutaneously to the chickens, with subsequent bleedings by heart puncture for assay of circulating antitoxin. Some of the chickens received two challenging doses of toxoid before the heart punctures were carried out on them.

Data on these experiments are shown in table 1.

TABLE 1

DATA ON CHICKEN EMBRYOS INJECTED WITH 34 LF OF DIPHTHERIA TOXOID INTRAVENOUSLY ON THE 12TH DAY OF INCUBATION AND CHALLENGED WITH 34 LF OF TOXOID AT VARIOUS INTERVALS FOLLOWING THE HATCHING

	Number of Embryos Injected	Number of Survivors at Time of First Challenge	Age at Time of First Challenge (Days)	Time between First Challenge and Bleeding (Days)	Age at Time of Second Challenge (Days)	Time between Second Challenge and Bleeding (Days)	Number of Chicken Bled
1. Toxoid Group	11	4	50	21			4
1. Control »	11	3	50	21			3
2. Toxoid Group	10	1	15	20			1
2. Control »	10	1	15	20			1
3. Toxoid Group	12	4	5	45			3
3. Control »	12	5	5	45			4
4. Toxoid Group	16	4	13		34	10	4
4. Control »	11	3	13		34	10	3

Note. — Measurable diphtheria antitoxin ($\geq$ 0.01 U/ml) was contained in the post challenge serum specimen of only one chicken of the toxoid group 2. (0.01 U/ml) and in the serum specimen of another chicken of the toxoid group 4. (0.01 U/ml). All the other chicken sera collected contained less than 0.01 U/ml of antitoxin.

As seen from table 1, measurable antitoxin was found in two chicken sera only out of the total of 23 bleedings taken. Furthermore, none of the sera deriving from the chickens of the control groups contained antitoxin. This somewhat unexpectedly found poor antibody response was supposed to be due to the relatively small dose of toxoid used for challenging of the chickens. Therefore, in the following experiments it was attempted to accomplish a better stimulation of the chickens by increasing the dose of toxoid and by the use of Freund adjuvant.

Series II. — This series consisted of 96 fertilized eggs.

Group 1. — On the 12th day of incubation, 23 eggs were injected by the intravenous route with 34 Lf of fluid toxoid, contained in 0.1 ml of diluent. As controls served 22 eggs, receiving 0.1 ml of diluent alone.

After the injections incubation was continued until hatching, which was reached in 5 cases in the toxoid group and in 8 cases in the control group. A challenging dose of 180 Lf of toxoid with Freund adjuvant, contained in a total volume of 0.8 ml, was then

given subcutaneously to all of the 13 chickens 50 days following the hatching. The chickens were bled by heart puncture 31 days after the challenge with toxoid.

Group 2. — On the 14th day of incubation, 26 eggs were injected with 34 Lf of fluid toxoid, as described above for group 1. As controls served 25 eggs.

After the injections incubation was continued until hatching, which was reached in 9 cases in the toxoid group and in 6 cases in the control group. A challenging dose of 250 Lf of toxoid with Freund adjuvant, contained in a total volume of 0.3 ml, was then injected subcutaneously to all of the 15 chickens 43 days following the hatching. Bleedings were taken by heart puncture 28 days later. To 7 chickens that survived, a second challenge with 250 Lf of fluid toxoid was given subcutaneously 126 days following the hatching.

Data on these experiments are shown in table 2.

From the data of table 2 it can be seen that all of the 14 chickens, to which toxoid had been administered intravenously on the 12th or 14th day of their embryonic life, had antitoxin in their circulation

TABLE 2

DATA ON CHICKEN EMBRYOS INJECTED WITH DIPHTHERIA TOXOID INTRAVENOUSLY ON THE 12TH OR 14TH DAY OF INCUBATION AND CHALLENGED WITH TOXOID AT VARIOUS INTERVALS FOLLOWING THE HATCHING

		Number of Embryos Injected	Number of Survivors at Time of First Challenge	Age at Time of First Challenge (Days)	Time between First Challenge and Bleeding (Days)	Antitoxin in the Bleeding taken after First Challenge (Units/ml)	Number of Survivors at Time of Second Challenge	Age at Time of Second Challenge (Days)	Time between Second Challenge and Bleeding (Days)	Antitoxin in the Bleeding taken after Second Challenge
1.	Toxoid Group	23	5	50	31	1-5, 1-5, 0.01, 0.1, 0.01				
	Control »	22	8	50	31	5, 0.1-1, 0.1-1, 0.01, 1-5, 0.01, 1-5, 0.1-1,				
2.	Toxoid Group	26	9	43	28	1-5, 5-10, 0.1-1, 0.1-1, 0.01-0.1, 0.1-1, 0.1-1, 0.1-1, 0.1-1,	4	126	10	10-20, 10-20, 1-5, 20-50,
	Control »	25	6	43	28	0.01, 1-5, 0.01, 0.01-0.1, 1, 0.01-1	3	126	10	5, 1-5, 0.1-1

at bleedings taken four weeks after a challenging larger dose of toxoid, given 6—7 weeks following the hatching. The same was true in the case of 10 out of the total of 14 chickens of the control groups, to which saline instead of toxoid had been administered during their embryonic life.

The relatively high content of antitoxin in the few sera taken 10 days following a second postnatal challenge with toxoid suggests that also the ability of the chickens to give a secondary response had remained unaltered by the fetal contact with toxoid.

DISCUSSION

The results of the experiments described, in which a single well-defined antigen antibody system was employed as an immunological tool, do not lend support to the hypothesis proposed by Burnet and Fenner, according to which foreign antigens, if introduced into an animal during the embryonic life, will thereafter be treated as autogenous, so that later exposure to the same antigen does not provoke an immunological response. Under the conditions studied in the present work, no such inhibitory effect on the ability of chickens to produce diphtheria antitoxin was seen. The prenatal contact with the antigen consisted of a single dose of diphtheria toxoid, administered intravenously to the embryos on the 12th or 14th day of the embryonic life. The differences in the experimental conditions make it difficult to compare the results obtained by various authors studying the problem of specific inhibition of the immune response. In those of the previous studies in which such a specific inhibitory effect definitely has been achieved on the ability of the animal to produce measurable circulating antibodies, the early contact with the antigen consisted of prolonged postnatal treatment of immature young animals with large doses of the antigen (8, 10, 12). On the other hand, in the light of the present knowledge it can be stated that so far no convincing experimental proof has been presented which would demonstrate that introduction of an antigen into the animal during the embryonic life exerts an inhibiting effect on the ability of the animal to produce measurable circulating antibodies against the same antigen later in life. Reports with negative results in accordance with those

obtained in the present work have been published by a number of authors (1, 2, 6, 12).

The studies so far published on the subject do not answer the question of the possible significance of quantitative interrelationships between the embryonic organism and the antigen introduced. It seems fully possible that a more prolonged and massive exposure of the embryonic organism to the antigen than hitherto achieved, would lead to a permanent and specific state of immunological unresponsiveness expressed as absence of circulating antibodies. The difficulties in carrying out studies on these quantitative aspects lay on the technical plane.

SUMMARY

Injection of chicken embryos by the intravenous route with a single dose of diphtheria toxoid on the 12th or 14th day of incubation did not inhibit the antitoxin response of the chicken, when exposed to the same antigen 6—7 weeks after hatching.

The results are discussed.

REFERENCES

1. AMBRUS J. L., et al.: Cited by BAUER J. A. PECKHAM, P. E., and OSLER, A. in Proc. Soc. Exp. Biol. & Med. 1956:92:714.
2. BAUER, J. A., PECKHAM, P. E., and OSLER, A. G.: Proc. Soc. Exp. Biol. & Med. 1956:92:714.
3. BEVERIDGE, W. I. M., and BURNET, F. M.: Spec. Rep. Ser. med. Res. Coun., Lond. No. 256, 1946.
4. BILLINGHAM, R. E., BRENT, L., and MEDAWAR, P. B.: Nature 1953: 172:603.
5. BURNET, F. M., and FENNER, F.: The Production of Antibodies, 2nd Edition, MacMillan, Melbourne 1949.
6. BURNET, F. M., STONE, J. D., and EDNEY M.: Austral. J. Exp. Biol. and Med. Sci. 1950:28:291.
7. BUXTON, A.: J. Gen. Microbiol. 1954:10:398.
8. CINANDER, B., and DUBERT, J. M.: Brit. J. Exp. Path. 1955:36:515.
9. COHN, M.: Methods in Medical Research 1952:5:275. Year Book Publishers, Chicago, Ill.
10. DIXON, F. J., and MAURER, P. H.: J. Exp. Med. 1955:101:245.
11. FRASER, D. T.: Trans. Roy. Soc. Canada Sec. 1931:25:175.
12. HANAN, R., and OYAMA, J.: J. Immunol, 1954:73:49.
13. WAGER, O., PALJAKKA, P., and SEPPÄLÄ, T.: Ann. med. exper. et biol. Fenniae 1955:33:125.

DIURNAL MITOTIC ACTIVITY IN THE CORNEAL EPITHE-LIUM OF A MOUSE[1]

EFFECT OF HYDROCORTISONE

by

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Upon investigating the effect of the hormones of the cortex of the suprarenal body on cell division in different tissues, it has been observed that hormones belonging to the glucocorticoid group inhibit mitotic activity in the epidermis (2, 3, 5, 8, 9). However, cortisone has no inhibitory effect on the mitotic activity of the small intestine (8) and the orbital gland (9) of a rat. Cortisone has been noted to augment mitotic activity in the corneal epithelium of a rat (7). The last-mentioned result seems contradictory, for in the light of the available evidence it should be expected that cortisone on the whole would tend to inhibit mitotic activity in tissues of ectodermal origin. For this reason, we deemed it appropriate to investigate the effect of hydrocortisone, a hormone belonging to the glucocorticoid group, on the mitotic activity of the corneal epithelium of mice as well as on the diurnal rhythm of the cell division through the day following the injection.

MATERIAL AND METHODS

Inbred C-strain[2] male mice were used as laboratory animals. Two months old (the maximum difference in age being five days),

the miec weighed 20—26 g. After weaning, the animals received the normal food mixture used in our laboratory; the feeding time was regularly 9—10 a.m. Water was given them ad libitum. Two weeks before the experiment was carried out, the animals were divided into three groups of twelve animals each. The tests took place on June 15 and 16. The light conditions in the animal shed were totally natural (the sun rose at 2.58 a.m. and set at 9.43 p.m. local time). Starting at 10.00 a.m., 1·mg of hydrocortisone (HYDRO-CORTONE ACETATE: MERCK & CO.)* was injected within a period of twelve minutes into the 36 animals. Between 12 o'clock noon of the same day and 10 a.m. the next day, one animal from each group of twelve was killed every two hours. Simultaneously, one animal from each of three control groups of the same size, kept in identical conditions, was killed at two-hour intervals. This was done by cutting the animals' neck with scissors. Immediately after the killings, the animals' eyes were enucleated, fixed for 24 hours in 4% neutral formaldehyde, and then transferred with maximum speed through an alcohol series and xylol into paraffin. Upon being set in the paraffin, the eyes were carefully placed in a horizontal plane. Series of 7 μ sections were made. Every third section was stained with Mayer's hemalaun, and the mitoses occurring in the corneal epithelium were counted. In order to eliminate any possible subjective influence, the slides were marked with code numbers, which were revealed to the investigators only after the entire material had been studied.

RESULTS

Table 1 shows the number of dividing cells at each two-hour interval in the corneal epithelium of the mice in the control group and in the group receiving injections of 1 mg hydrocortisone. A graph has been drawn (Fig. 1) on the basis of the average mitotic activity. Among the control animals, cell division reaches a maximum at 8 a.m., when the count averages 417.3±107.7, and a minimum at 8 p.m., when the count is 14.3±4.3. Among the animals treated with hydrocortisone, almost equally high mitotic activity occurs at 4 a.m. (126.0 ± 25.1) and 8 a.m. (118.7 ± 8.7).

TABLE 1

THE NUMBER OF DIVIDING CELLS AT EACH TWO-HOUR INTERVAL IN THE EXAMINED
CORNEAL EPITHELIUM

Time	Control	Mean of Control ±S.E.	Hydrocortisone-Treated	Mean of Hydro-cortisone ±S.E.
12 noon	56 28 121	68.3 ± 27.6	36 50 116	67.3 ± 24.8
2 p.m.	106 189 68	121.0 ± 34.3	67 167 51	95.0 ± 35.6
4 p.m.	40 135 40	71.7 ± 31.7	10 15 73	32.7 ± 20.2
6 p.m.	20 75 35	43.3 ± 16.4	6 24 41	23.7 ± 10.2
8 p.m.	14 7 22	14.3 ± 4.3	12 22 11	15.0 ± 3.5
10 p.m.	29 13 4	15.3 ± 7.3	4 5 4	4.3 ± 0.1
12 mid-night	32 14 26	24.0 ± 5.3	54 11 32	32.3 ± 12.4
2 a.m.	100 112 153	121.7 ± 16.1	6 104 27	45.7 ± 29.8
4 a.m.	94 193 163	150.0 ± 29.3	176 154 48	126.0 ± 25.1
6 a.m.	139 217 141	165.7 ± 25.7	113 51 72	78.7 ± 18.2
8 a.m.	605 232 415	417.3 ± 107.7	103 133 120	118.7 ± 8.7
10 a.m.	112 186 166	154.7 ± 22.1	49 73 64	62.0 ± 7.0

Fig. 1. — The average mitotic activity in the corneal epithelium in the control group and in the group receiving hydrocortisone.

The least number of mitoses in this group is to be observed at 10 p.m., when the mean is 4.3 ± 0.1.

As we have noted previously, mitotic activity in the intact corneal epithelium was greater between the hours 2 a.m.—2 p.m. than 2 p.m.—2 a.m. (10). The mean frequency of cell division among untreated animals between 12 o'clock noon and 10 p.m. is 55.7, while between 12 o'clock midnight and 10 a.m. it is 172.2 The difference between these mean values $\pm$ standard error is 116.5 ± 35.2. With the t-test, we obtain as the level of significance of the difference between the means $0.005 > P > 0.001$ (d.f. $= 34$, $t = 3.315$). The mean mitotic frequency among animals treated with hydrocortisone between 12 noon and 10 p.m. is 39.7, whereas between 12 midnight and 10 a.m. it is 77.2. The difference between these means $\pm$S.E. is 37.5 ± 15.4. Continuing to apply the t-test, we obtain as the level of probability of the difference $0.02 > P > 0.01$ (d.f. $= 34$, $t = 2.434$). Among the untreated animals, the difference between the periods of maximum and minimum activity is statistically significant, whereas, again, among the animals receiving hydrocortisone, the difference between these periods is also significant, though on a lower level.

The mean mitotic frequency in the hydrocortisone group after the injection is 38.6 during the period 12 noon—12 midnight,

TABLE 2

THE DIFFERENCE BETWEEN THE MEANS OF MITOTIC ACTIVITY OF THE GROUPS
DURING THE PERIODS 12 NOON—12 MIDNIGHT AND 2 A.M.—.0 A.M.

Time	Mean of Controls	Mean of Treated	Difference between Means	S. E. of Differ-ence	Degree of Freedom	t	p
2 a.m.— 10 a.m.	201.8	86.2	115.6	37.3	28	3.097	< 0.005 > 0.001
12 noon— 12 midnight	51.1	38.6	12.5	13.9	40	0.899	< 0.40 > 0.30

TABLE 3

THE DIFFERENCE BETWEEN THE MEANS OF MITOTIC ACTIVITY OF THE GROUPS
AT EACH TIME INTERVAL DURING THE PERIOD 2 A.M.—10 A.M.

Time	Mean of Controls	Mean of Treated	Difference between Means	S.E. of Differ-ence	Degree of Freedom	t	p
2 a.m.	121.7	45.7	76.0	33.2	4	2.287	<0.1 >0.05
4 a.m.	150.0	126.0	24.0	49.2	4	0.488	<0.70 >0.60
6 a.m.	165.7	78.7	87.0	31.5	4	2.765	<0.1 >0.05
8 a.m.	417.3	118.7	298.6	108.2	4	2.760	<0.1 >0.05
10 a.m.	154.7	62.0	92.7	23.3	4	3.982	<0.02 >0.01

while in the control group the figure is 51.1. As may be seen in
Table 2, the difference between these means is not, however,
statistically significant. During the period 2 a.m.—10 a.m., the
mean of mitotic activity is 201.8 in the control group and 82.2
in the hydrocortisone group. Table 2 shows, further, that this
difference is statistically significant. As we have noted in the fore-
going, the mitotic frequency is greater during the latter period
than the former in both the control and hydrocortisone groups. It
is therefore obvious that the inhibitory effect of hydrocortisone
on mitotic activity does not become apparent until the active
period in the diurnal rhythm is reached. Although during the
period between 2 a.m. and 10 a.m. the mean mitotic frequency is
statistically significantly lower in the hydrocortisone group than

in the control group, this difference cannot be perceived upon statistically comparing the groups together at each time interval into which the period is divided (Table 3). The most significant difference occurs at 10 a.m., but the decrease in mitotic activity at 2 a.m., 6 a.m. and 8 a.m. may also be regarded as statistically significant, if on a lower plane. At 4 a.m. no statistically significant decline in cell division can be perceived among the animals receiving hydrocortisone as compared with the control animals.

DISCUSSION

Our results show that hydrocortisone, intraperitoneally injected (1 mg), has an inhibitory effect on the mitotic activity in the corneal epithelium of mice during the active period of the diurnal cycle. The diurnal rhythm continues during the twenty-four hours following the injection, but the difference between the maximum and minimum activity diminishes conspicuously as compared to the corresponding difference in the control group. When the diminishing tendency prevails during the normal diurnal cycle of mice, hydrocortisone cannot be perceived to have any statistically significant inhibitory effect. As is known, hydrocortisone dissolves relatively poorly in tissue liquids (4) and this may also explain the fact of the effect showing only after a relatively long latent period. Still, Chaudhry et al. (3), upon intraperitoneally injecting 1 mg hydrocortisone into mice during the active period of the diurnal cycle, could observe a statistically significant inhibition of mitotic activity in the epidermis of the ear tip within as short a time as four hours after the injection. In our own material, a clear inhibition does not become evident before 15.5 hours have passed, when the active period in the diurnal mitotic cycle likewise starts.

Sigelman *et al.* (7) removed the right eye of rats and thereafter gave 5 mg cortisone acetate injections i.m. over a stretch of five days. Following the fifth injection, the animals were killed and the left eye was removed. A comparative count of the mitoses in the right and left eyes revealed an increase of 24% in the left. If the hypophysis was removed and the animals were treated in the manner described in the foregoing, no difference what so ever could be detected between between the respective mitoses. Simply the

removal of the hypophysis, on the other hand, caused a decrease in mitotic frequency. Removal of the suprarenal body and parenteral administration of ACTH, however, had no effect on the mitotic activity in the corneal epithelium of rats. The technique used by the investigators mentioned does not seem incontrovertibly reliable, for certain other researchers (6) have noted a considerable difference between the two eyes of the same animal in respect to their mitotic activity when both organs have been removed simultaneously. Furthermore, in the work of Sigelman *et al.* there is no mention of the time of day at which the mitotic activity was determined and whether it was determined for each eye at exactly the same time of day. Nor did they take into account the relation of the time of killing to the diurnal cell division cycle of normal animals, a factor that in the light of the results reported in the present paper obviously has considerable importance. In addition, a quantity so greatly dependent, evidently, on merely external factors as mitotic activity cannot be considered a stabile quantity even in the same animal upon different days being involved in respect to the time of determining the mitotic frequency (1). Sigelman *et al.* do not, however, mention having standardized the external factors in any way during the time consumed in carrying out their study.

Our results lend support to the view that glucocorticoids inhibit the mitotic activity in tissues of ectodermal origin, as has previously been demonstrated in the case of the epidermis (2, 3, 5, 8, 9), and the corneal epithelium does not constitute an exception, as Sigelman et al. (7) assert on the basis of their work.

SUMMARY

The study deals with the mitotic activity in the corneal epithelium of inbred C-strain male mice, aged two months, during the 24-hour period following intraperitoneal injections of 1 mg hydrocortisone. Thirty-six animals were treated and 36 untreated. Cell division proved to be more active in the early morning and the forenoon than in the afternoon and at night in the case of both control and treated animals. Hydrocortisone reduces the mitotic frequency in the corneal epithelium of mice. The inhibitory effect becomes evident during the active period of the diurnal cycle.

REFERENCES

1. BULLOUGH, W. S.: Proc. R. Soc. B. 1948:*135*:212.
2. BULLOUGH, W. S.: J. Endocrinol. 1952:*8*:265.
3. CHAUDHRY, A. P., F. HALBERG, and J. J. BITTNER: Proc. Soc. Exper. Biol. Med 1956:*91*:602.
4. DRILL, V. A.: Pharmacology in Medicine, McGraw-Hill, 1954.
5. GREEN, H. N., and F. N. GHADIALLY: Brit. Med. J. 1951:*1*:496.
6. KAUFMANN, B. P., H. GAY, and A. HOLLAENDER: Anat. Rec. 1944: *90*:161.
7. SIGELMAN, S., C. H. DOHLMAN, and J. S. FRIEDENWALD: Arch. Ophthalmol. 1954:*52*:751.
8. STUDER, A.: Bull. Schweiz. Akad. Med. Wiss. 1952:*8*:60.
9. TEIR, H., and A. ISOTALO: Ann. Med. Exper. Biol. Fenn. 1953:*31*:171.
10. VASAMA, R., and RITVA VASAMA: Acta Anat., in press.

DETOXICATION CAPACITY OF RAT LIVER AND INTESTINE DURING PROLONGED FEEDING OF CINCHOPHEN

by

E. AANTAA, S. AANTAA and K. HARTIALA

Previous studies have shown that cinchophen diminishes the secretion of mucus by the duodenal and pyloric mucosa (3, 8). This effect has been connected with the glucuronide synthesis carried by the organism. On the other hand it has been found that the hepatic glucuronide synthesis is not effected during cinchophen toxication (6). After this the observation has been made that in addition to the liver, also the mucous membrane of the gastro-intestinal tract posesses the same property to conjugate glucuronides (1, 2). The purpose of this investigation was to find out whether prolonged feeding of cinchophen causes changes in the gastrointestinal detoxication properties.

MATERIAL AND METHODS

Male rats (Wistar-breed) weighing c. 150—200 gr. were used in the experiments. Cinchophen was given through a thin polythene tube introduced in the stomach. Two separate experimental groups were used.

The first group including 10 rats received a 2% cinchophen solution made with 2 per cent Na_2CO_3 the daily dose being 100 mg per kilo body weight and carried over a period of 55 days. Later a stronger cinchophen solution was used (3% cinchophen dissolved

in a 2.5% sodium carbonate water solution). With this solution experiments were continued over a period of 25 days the daily dose also being increased to 150/kg body wt. The weight was controlled weekly and the dose corrected accordingly.

After this feeding period the animals were killed by a blow on the head. The liver and mucosal specimens from the stomach and intestines were removed and analysed to their glucuronide conjugation capacity according to the method previously described in detail (7, 5).

The second group included originally 14 rats; 11 of these survived to the final analyses. These animals recieved from the very beginning the stronger cinchophen solution. Daily feeding of 150 mg cinchophen per kg body weight was carried on over a period of 35 days. The analytical procedures were the same as in the first part. A total number of 21 untreated rats served as a control group.

RESULTS AND CONCLUSIONS

In group 1 the following results were obtained:

TABLE 1

EFFECT OF PROLONGED FEEDING OF CINCHOPHEN ON THE IN VITRO SYNTHESIS OF GLUCURONIDES. RESULTS EXPRESSED AS γ O-AMINOPHENOLGLUCURONIDE PRODUCED PER 100 MG (DRY WEIGHT) TISSUE IN 90 MINUTES. THIS GROUP INCLUDING 10 RATS RECEIVED 100 MG CINCHOPHEN PER KILO BODY WEIGHT DAILY OVER THE PERIOD OF 55 DAYS AND 150 MG DAILY OVER THE PERIOD 25 DAYS

Tissue		Control			Cinchophen-Treated	
			StD	StE		StD	StE
Liver	55.1	17.6	5.6	73.0	26.9	8.5
Mucosa duodenal	149	98.6	31.1	97.2	53	16.8
gastric	43.0	23.7	11.6	49	32.4	16.2
pyloric	65.7	21.2	10.6	107.3	33.6	19.2

A great variation in individual animals is evident. This is not mainly due to the method itself since duplicate samples taken from the same area and animal usually give a satisfactory agreement. The size of tissue specimens also has been standardized; the optimal seize corresponding to 10 mg in dry weight.

The results listed in the table show no significant difference in the cinchophen fed and control groups. The lower results obtained

from the duodenal mucosa in the cinchophen treated group is not statistically significant.

TABLE 2

EFFECT OF PROLONGED FEEDING OF CINCHOPHEN ON THE IN VITRO SYNTHESIS OF GLUCURONIDES. RESULTS EXPRESSED AS γ O-AMINOPHENOLGLUCURONIDE PRODUCED PER 100 MG. RATS RECEIVED 150 MG CINCHOPHEN PER KILO BODY WEIGHT DAILY OVER THE PERIOD OF 35 DAYS

Tissue		Control			Cunchophen-Treated		
			StD	StE		StD	StE
Liver		82.1	35.8	14.6	121	27	9
Mucosa	duodenal	295	106	43	248.3	70	22
	colon	103.5	28.8	11.7	76.9	38.2	12
	gastric	29.9	5	3 5	27.6	1.8	0.9
	pyloric	56.4	20.4	9	62.4	31.2	11

The second group included younger animals which during the experiment gained some weight. Whereas the former animals lost on average 20 gr in their weight during the treatment this group showed on average increase of 60 gr after the cinchophen feeding period. No studies are available to allow conclusions to be drawn about the glucuronide forming capacity during growth and at various ages. It might be possible that this capacity still increases during the growth period. At the time of birth it is not the same as in adult as has been observed in our previous studies (4).

Cinchophen feeding has not effected the glucuronide synthesis capacity either in this group. By other words this means that during prolonged treatment of this toxic agent the ability of the organism to conjugate this substance to a glucuronide is not exhausted. This synthesis is carried on nearly as usual. In this respect these findings confirm our previous studies in guinea pig and dog which showed that the liver is able to synthetize glucuronides during cinchophen toxication (6).

Blood counts made during the experiments showed no other changes in the red or leucocyte counts than an elevated monocyte amount (average 9.4 per cent) which is higher than reported for normal values. The hemoglobin amounted to 10.9/100.

It has been shown that the mucus secretion is reduced during cinchophen toxication. The present observations lend some more support to our previous speculations about the mechanism of this

reduction. Our recent studies have shown that cinchophen is combined to a glucuronide in the gastrointestinal tract (9). Cinchophen might cause such a strong burden on the secretion elements of the mucous membrane that they cannot simultaneously carry on with their usual secretory functions. Whether this also means that the same glandular and cellular elements carry on both functions, detoxication and secretion, is not settled with these findings. This possibility still remains and if so these functions would be competitive in nature.

SUMMARY

Cinchophen was given orally to rats in doses of 100 mg and 150 mg per kilo body weight. No significant reduction was observed in the capacity of the liver or mucous membrane of the gastrointestinal tract to conjugate glucuronides in vitro. These results tend to support our previous view about the competitive nature of the intestinal mucus secretion and glucuronide synthesis.

REFERENCES

1. HARTIALA, K. J. W.: Acta Physiol. Scand. 1954:*31*, suppl. 114:20.
2. HARTIALA, K. J. W.: Ann. Med. Exp. Fenn. 1955:*33*:239.
3. HARTIALA, K. J. W., A. C. IVY, and M. I. GROSSMAN: Amer. J. Physiol. 1950:*162*:11.
4. HARTIALA, K. J. W., and M. PULKKINEN: Ann. Med. Exp. Fenn. 1955:*33*:246.
5. HARTIALA, K. J. W., and SIRKKU RONTU: Ann. Med. Exp. Fenn. 1955:*33*:213.
6. HARTIALA, K. J. W., and L. TELIVUO: Ann. Med. Exp. Fenn. 1955:*33*:219.
7. LEVVY, G. A., and D. E. STOREY: Biochem. J. 1949:*44*:295.
8. MAGEE, D. F., K. S. KIM, and A. C. IVY: J. Appl. Physiol. 1951:*3*:736.
9. SAVOLA, P., LEIKKOLA, and K. J. W. HARTIALA: In press.

INHIBITION OF CHLORPROMAZINE-CAUSED FATTY LIVER WITH CHOLINECHLORIDE IN THE WHITE RAT

by

ARMO HORMIA[1] and MARTTI HORMIA

In an earlier paper (4) the effect of chlorpromazine and reserpine on liver parenchyme in the white rat was investigated. Neither reserpine nor combined reserpine-chlorpromazine could be shown to affect the liver. Also in animals receiving a so large chlorpromazine dosage from the very beginning of medication that they died of it in a short time, the histologic picture of the liver proved to be normal. In animals receiving slowly increased doses of chlorpromazine, and which thus became well adapted to a massive medication, fat infiltration could be seen in 14 of 18 cases. Besides this, necrosis or other regressive changes were seen in 10 cases. Only in one case the histologic picture of the liver proved to be normal. In the light of these results, the hypothesis of the significance of an insufficiency in the function of the lipotropic factors in the etiology of chlorpromazine-caused liver parenchyme changes was presented.

The purpose of the experiments presented hereunder was to investigate the relation between lipotropic factors and the liver parenchyme lesions caused by chlorpromazine. The possibility to avoid these changes by the means of simultaneous application of cholinechloride was studied.

MATERIAL AND METHODS

The effect of simultaneous cholinechloride medication on the occurrence
of liver parenchyme lesions during chlorpromazine medication was studied
by 10 female white rats. The animals were of the same age as those used for
the former investigation with chlorpromazine alone. Also in other respects
the circumstances and the methods were the same (cf. 4). The drugs were
given subcutaneously. Cholinechloride was used in a dose of 20 mg/kg
body weight daily. Chlorpromazine was given on the first day 5 mg/kg
body weight and the dosage was increased daily with this same amount.
The medication was continued during 25 days, up to the dosage of 125
mg/kg body weight. After this the rats were killed. The animals were
weighed before the medication was started and again after death, also the
liver was weighed then. To demonstrate the possible changes in the weight
of the liver during the medication, the body and the liver were weighed by
9 control rats.

A specimen of the liver for microscopic study was taken immediately
after death. The possible occurrence of fat was studied by cutting 10 μ
frozen sections and by staining them with the Sudan III method. After
this the specimen was embedded in paraffin and 5 μ sections were cut and
stained with the van Gieson method. For glycogen sections were stained
also with the Best's carmine method.

RESULTS

In the 9 control rats, the weight of the liver was 3.8% of the
total body weight. In the 10 experimental rats the mean body
weight before medication was 279 g. Thus the normal weight
of the liver should have been about 10.7 g. In reality, however, it
was 18.4 g on an average. This means that the liver had gained in
weight by 73% during the combined cholinechloride-chlorpro-
mazine medication. The total body weight had increased by 12 g on
an average, thus for the main part due to the increase of the weight
of the liver.

By studying the liver microscopically slight degenerative chan-
ges in the liver parenchyme cells could be seen in all cases: a more
or less granular appearance of the cytoplasm and slight pycnosis
of the nuclei. In one case a little necrotic region near the capsule was
obtained. In all cases the glycogen content of the tissue was nor-
mal. In four cases no fat could be seen in the cells by the Sudan III
method. In five cases several small Sudan-positive fat droplets were
obtained in many of the liver cells and here and there some single,
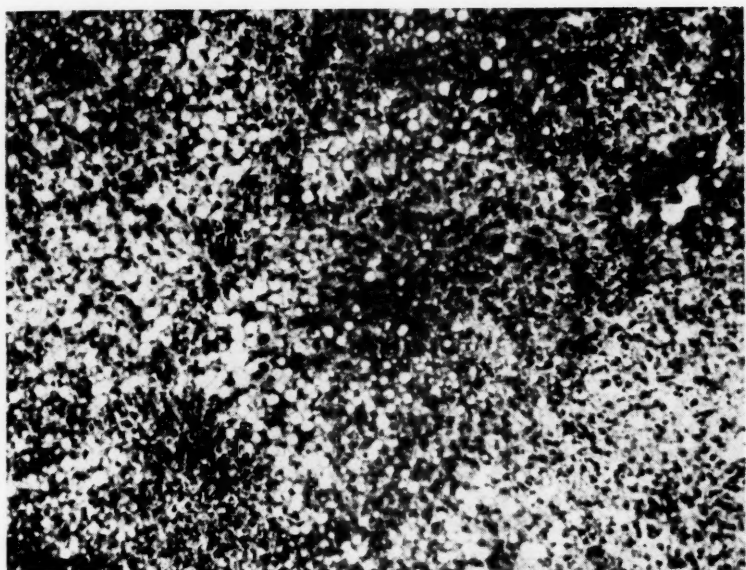large fat droplets. In one case it could be considered to be a ques-

Fig. 1. — Photomicrograph of the rat liver showing extensive fat infiltration caused by prolonged, massive chlorpromazine medication. Van Gieson stain, × 360.
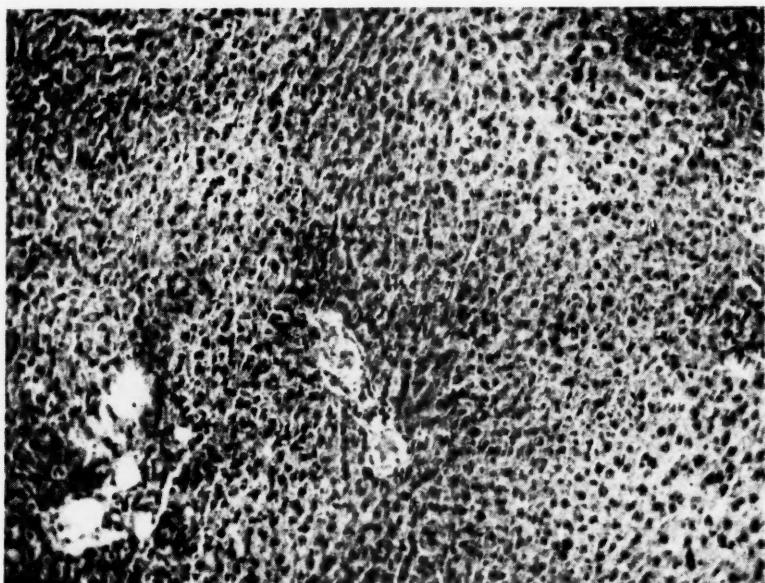

Fig. 2. — Photomicrograph of the rat liver in a typical case of combined cholinechloride-chlorpromazine medication. Some pyknosis of the nuclei is seen. Van Gieson stain, × 360.

tion of a mild case of fatty degeneration: many cells were seen to be filledby small fat droplets and here and there some single, large fat droplets were obtained.

DISCUSSION

Table 1 shows the results of the present investigation compared with the former results with chlorpromazine alone. It can be seen that the simultaneous cholinechloride application has essentially decreased the occurrence and degree of fat infiltration during prolonged, massive chlorpromazine medication. Moderate or pronounced fatty liver was obtained in the group of combined choline-chloride-chlorpromazine medication only in 1 case of 10 (=10%), in the group of chlorpromazine alone this was the case in 11 cases of 18 (= 61%).

TABLE 1

OCCURRENCE OF FATTY LIVER AND DEGENERATIVE CHANGES OF THE LIVER DURING CHLOR PROMAZINE MEDICATION AND COMBINED CHOLINECHLORIDE-CHLORPROMAZINE MEDICATION.

0 = no changes, + = mild, ++ = moderade, +++ = pronounced changes.

In the table are also seen the changes of the body weight and that of the liver in each group

	Fatty Liver				Total	Degenerative Changes of the Liver				Change of the Body Weight	Change of the Weight of the Liver
	0	+	++	+++		0	+	++	+++		
Chlorpromazine medication	3	4	7	4	18	8 —		8	2	—16%	+24%
Combined choline-chloride-chlorpromazine medication	4	5	1	—	10	— 10	—		—	+ 4%	+73%

The simultaneous cholinechloride medication intensified the functional hypertrophy of the liver already seen in the group of chlorpromazine alone. The general condition of the animals remained somewhat better in the group of the combined medication, as is seen by comparing the changes in the body weight in the two groups. Indeed the rats in the group of combined medication seemed to have no harm from the medication.

These results are in accordance with the hypothesis that the fat infiltration of the liver during a prolonged chlorpromazine medication were due to an insufficiency of the function of the lipotropic

factors. On the other hand, the deficiency of lipotropic factors has not been considered to have any essential role in the etiology of liver parenchyme changes other than fat infiltration (1, 2, 3). In accordance with this the occurrence of degenerative changes of the liver during chlorpromazine treatment, viz. a granular degeneration of the cytoplasm, was only slightly, if at all influenced by the simultaneous application of cholinechloride. These changes were of lesser degree but occurred more frequently in this group.

SUMMARY

The effect of cholinechloride on the occurrence of fat infiltration of the liver during a prolonged, massive chlorpromazine medication was studied by 10 white rats. The results were compared with those of chlorpromazine alone. It was shown that the simultaneous application of cholinechloride essentially reduced the occurrence and amount of fatty liver during chlorpromazine medication. Other, degenerative parenchyme changes of the liver, on the contrary, were not essentially influenced by cholinechloride. These results were considered to be in accordance with the hypothesis that the fat infiltration of the liver caused by chlorpromazine medication were due to an insufficiency of the function of the lipotropic factors.

REFERENCES

1. BEST, C.: Lancet 1934:*1*:1155, 1216, 1274.
2. BEST, C.: Science 1941:*94*:524.
3. HOFF, F.: Klinische Physiologie und Pathologie. Stuttgart 1957.
4. HORMIA, M., HORMIA, A., and HAKOLA, P.: Ann. Med. exp. Fenn. 1957:*35*:316.

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THE NEUROSECRETORY MATERIAL OF THE NEURO-HYPOPHYSIS UNDER EXPERIMENTAL STRESS SITUATION

by

E. KIVALO and H. ARKO

The neurosecretory material, demonstrable by selective histo-logical means in the hypothalamico-neurohypophyseal system, is related to the neurohypophyseal hormones vasopressin (= anti-diuretic substance) and oxytocin. Numerous observations support the opinion that the neurosecretory material is produced by hypo-thalamic neurons in supraoptic and paraventricular nuclei and passes along nerve fibres in the infundibular process over to the posterior pituitary. It has further been assumed that the neuro-hypophysis is not a gland in the strict sense but only an organ of storage and release into the blood stream for the hormones formed in the hypothalamus (13).

The matter is peculiar enough as such, but it becomes even more interesting owing to observations which indicate that the neuro-hypophyseal hormones might be closely related to the control of ACTH release from the adenohypophysis (16, 12, 6, 7, 15). In spite of many attempts, it has not been definitely demonstrated that endo-genous vasopressin or oxytocin would be the natural mediator of the ACTH discharge, although administration of vasopressin or oxytocin produces a marked ACTH release (6). This effect has recently been attributed to a contaminant in commercial prepara-tions of vasopressin (2, 14). These last authors, Saffran, Schally

and Benfey, found in posterior pituitary extracts a third substance distinct from vasopressin and oxytocin. Like these, it is probably elaborated within neurosecretory cells of the hypothalamus and stored in the posterior pituitary. This substance clearly stimulates in vitro the ACTH discharge from rat pituitary.

If this substance (CRF = corticotrophin-releasing factor) indeed occurs in the neurosecretory material, then it can be expected that stressed animals would show a depletion of this material in histological examinations. Such observations have been made (10, 11, 1). These data evidently suggest a participation of the neurosecretory material in the pituitary-adrenal response to stress. On the other hand also evidence in opposite directions has been reported (9, 3).

In our experiments concerning neurosecretion and diuresis (5) we could not observe that the neurosecretory material would be as easily influenced as has been postulated. Therefore, we extended our experiments to include studies as to 1) whether we might notice any depletion of neurosecretory material from the posterior pituitary during experimental stress situations and 2) if so, whether there is any anatomical evidence of its potentially augmented passage into the circulation.

MATERIAL AND METHODS

The series consisted of 44 white rats, half and half of both sexes. 17 of them were control animals. The weight varied between 135—310 g. The control animal group used in the different experiments was in every case of the same sex and the animals were of approximately equal weight. All animals were on normal feeding and they were allowed to drink ad lib. Furthermore, 12 white female mice were used for the experiments, six of them being controls. Their weight varied between 15—20 g.

The test animals were subjected to stress by the following methods:

Ten rats were trasferred from the animals stables to the laboratory room several storeys higher up. The animals were killed within one hour swift decapitation, while the remaining animals were kept in a cage on the same table on which the decapitation was performed. The control animals were killed in the animal

stables, taking them from their own cage one at a time and performing rapid decapitation in the adjoining handling room.

Six mice were transferred from the animal stables to the laboratory and disturbed with flicker light during 24 hours continuously. The flicker frequency was three flashes per second with a 40 watt bulb placed immediately above the cage. The six control mice were kept in the same room similarly for 25 hours, but they were protected from the flicker. The decapitation of all animals was carried out rapidly in succession in the same room.

The following experiments were performed with altogether 17 rats: Four rats were transferred from the animal stables to the test room where they were subjected to strong flicker light with simultaneous strong noise in the room. One group was kept in the test for 10 minutes and the other group for 30 minutes. — Two rats were kept at —7°C for 10 minutes and one rat for 30 minutes; one rat was kept continously at +4°C for 3½ months. Two rats were stabbed in the tail with an injection needle about 20 times in succession during 1—2 minutes; two rats were completely immobilized and subcutaneously injected with 0.5 ml of Bouin's solution. One of them was killed 10 minutes and the other one 30 minutes after the injection. Four rats were injected intraperitoneally with 5% formalin; two of them died spontaneously within 2—15 minutes and the other two were killed 15 minutes after the injection. As a last test, one rat was put into the cage of a cat who lacerated it and dragged it around for five minutes. The rat, still alive, was then taken away from the cat and rapidly killed. All animals in the tests described above were killed swiftly by decapitation. The control animals were killed at comparable times in the animal stables by decapitation.

In each case the hypophysis was removed immediately; Bouin's solution was used as a fixing agent. The fixing time was 24 hours. The preparations were treated in the usual way, embedding them in paraffin. Part of the preparations were frozen after fixing, and stained. The thickness of the cuts varied between 5—20 micra. However, the thickness of 5 micra was used in general. The staining methods employed were Gomori's chrome-alum-hematoxylin-phloxin method and Gomori's aldehyde-fuchsin method, preceded by an oxydation phase. However, no actual comparative study between these two methods has been performed in this investigation.

RESULTS

In spite of the stress effects of various type and of their varying duration, no depletion of neurosecretion in the posterior pituitary could be observed in any individual case by the methods employed, as compared to the control series. However, in some singularly strong stress situations, such as at the intraperitoneal injection of formalin, distention of the capillaries in the posterior pituitary could be observed. In these cases, too, neurosecretory material remained unchanged. Also, no tendency was observable that such material would have been transferred intravascularly (Figs. 1, 2, 3).

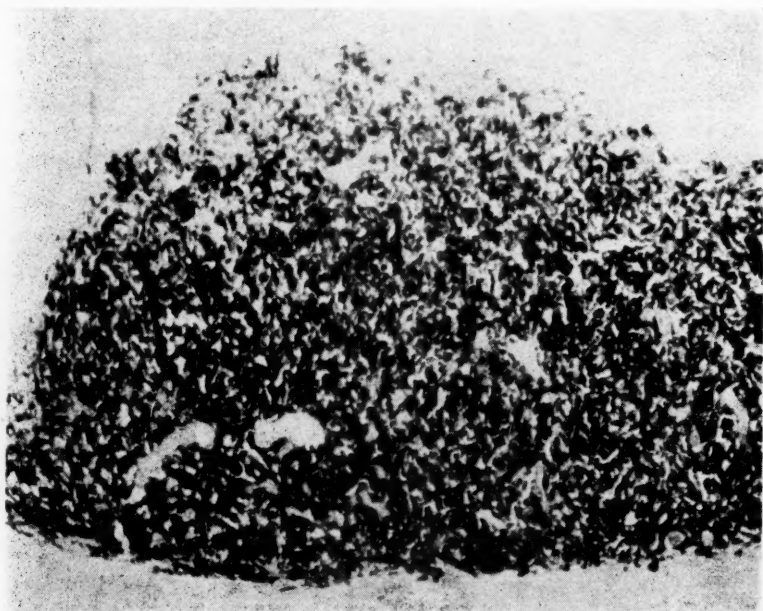Fig. 1. — The posterior pituitary of a rat after intraperitoneal injection of 2 ml 5 per cent formalin. Clearly visible dilatation of the capillaries. Aldehyde-fuchsin method. 80 ×.
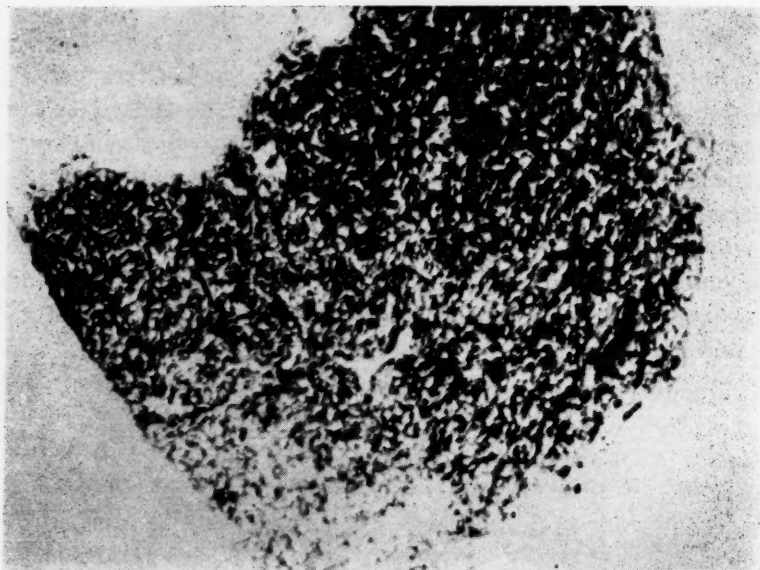
Fig. 2. — The posterior pituitary of a control rat. Aldehyde-fuchsin method.
80 ×.
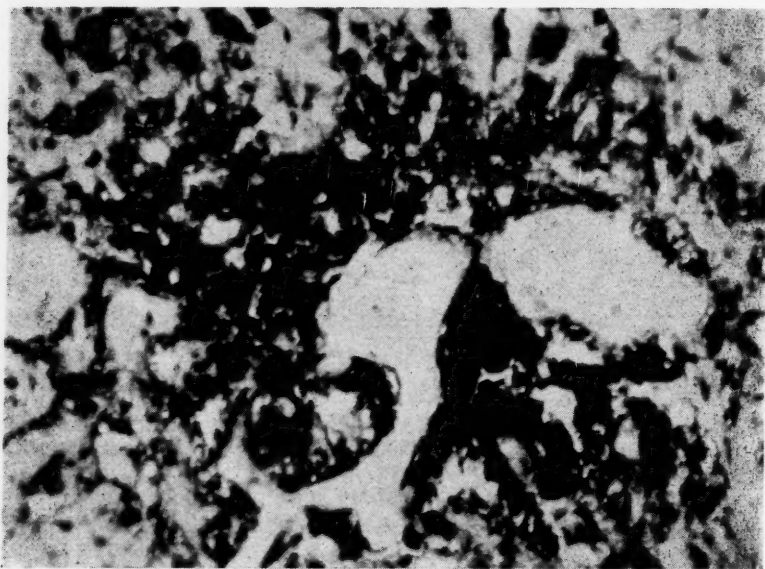
Fig. 3. — Photomicrograph of the same posterior lobe as in fig. 1. Intravascularly no neurosecretory material is visible. 320 ×.

DISCUSSION

Verney (16) showed that unpleasant stimuli will induce a release of antidiuretic hormone from the neurohypophysis. According to Rothballer (10, 11) and to Barrnett (1), it can be expected histologically as well that the neurosecretory material will be depleted in the posterior lobe after similar situations. However, we have not been able with our methods to observe any similar phenomenon. Our observations are therefore in aggreement with the investigations of Rennels *et al*, (9) who could not find any changes in the neurosecretory material of the posterior lobe after electric shock treatment of rats. De Groot (3), too, concludes from his histological investigation that surgical stress had no consistent effect upon the neurosecretory material and that the neurosecretory system bears no direct relationship to the adenohypophysial function. According to O'Connor and Verney (8), it is difficult to define the variations of antidiuretic hormone in physiological situations, since the physiologically required quantity of this hormone is very small. Against this background, and also because the conception has been presented (4) that the neurosecretory material is merely the so-called carrier part of antidiuretic hormone, it is not likely that it might disappear so abruptly even though hormone itself would be transferred into the circulation as a result of alarm reaction.

SUMMARY

With the aid of experiments with rats and mice, the behaviour of the neurosecretory material in the posterior pituitary under various stress states was studied. It is noted that in spite of various stimuli and of varying duration of these no differences could be observed in comparison to the control material. In strong stress states distension of the capillaries in the posterior pituitary was noticed but even then no depletion of the neurosecretory material could be observed, nor any transfer of the same into the capillaries. According to the authors, the employed histological methods are not sufficient to indicate potential neurosecretory response to stress.

REFERENCES

1. BARRNETT, R. J.: Endocrinology 1954:*55:*484.
2. GUILLEMIN, R., and HEARN, W.: Proc. Soc. Exper. Biol. & Med. 1955:*89:*365.
3. DE GROOT, J.: Anat. Rec. 1957:*127:*2.
4. HILD, W., and ZETLER, G.: Z. exper. Med. 1953:*120:*236.
5. KIVALO, E., and ARKO, H.: Ann. med. exp. et biol. Fenniae, 1957 in press.
6. MARTINI, L., and MORBURGO, C.: Nature 1955:*175:*1127.
7. MIRSKY, I. A., STEIN, M., and PAULISCH, G.: Endocrinology 1954:*55:*28.
8. O'CONNOR, W. J., and VERNEY, E. B.: Quart. J. of Exper. Physiol. 1942:*31:*393.
9. RENNELS, E. G., RUSSELL, G. V., and DRAGER, G. A.: Anat. Rec. 1955:*121:*355.
10. ROTHBALLER, A. B.: Anat. Rec. 1953:*115:*21.
11. ROTHBALLER, A. B.: Acta Neurovegetativa 1956:*13:*179.
12. SCHARRER, E., and FRANDSON, R. D.: Anat. Rec. 1954:*118:*350.
13. SCHARRER, E., and SCHARRER, B.: v. Möllendorfs Handbuch d. mikr. Anat. d. Menschen, Bd. VI/5:953. Heidelberg 1954.
14. SAFFRAN, M., SCHALLY, A. V., and BENFEY, B. G.: Endocrinology 1955:*57:*439.
15. SOBEL, H., LEVY, R. S., MARMORSTON, J., SCHAPIRO, S., and ROSENFELD, S.: Proc. Soc. Exp. Biol. and Med. 1955:*89:*10.
16. VERNEY, E. B.: Proc. Roy. Soc. 1947:*135:*27.

ACID PHOSPHATASE ACTIVITY IN THE CEREBELLUM OF THE RAT

by

E. KIVALO and L. LEHTINEN

Comparatively few investigations by histochemical means have been made of the acid phosphatase occurring in the central nervous system. Mainly, the activity of this enzyme has been shown in such places where it was expected to be higher than normal, e.g., in the chorioid plexus and in the magnocellular nucleic cells of the hypothalamus on account of the secretory activity occuring in them (1, 2, 5). Some investigations have also been performed with regard to the said activity at comparison between different parts of the central nervous system in the region of the hematoencephalic barrier, and in different parts of the cortex on the other hand (4, 7). In the different parts of the cerebellar hemisphere the acid phosphatase has been demonstrated in connection with a work concerning comparison of different methods (6).

The purpose of the present work was to determine acid phosphatase activity in the various parts of the cerebellum, primarily as a comparison in this respect between different cell types, and to compare the activity occurring in them to that in the chorioid plexus of the fourth cerebral ventricle.

MATERIAL AND METHODS

The series consisted of three male and three female white rats, which were killed by swift decapitation. Immediately thereafter the brains and the medulla oblongata were extracted with the greatest

possible care. The preparations were immersed in fix solution, using 4 per cent formalin solution to this purpose. The acid phosphatase was shown by the modification of Gomori's method presented by Eränkö (3). The thickness of the cuts was 20 micra, the incubation time 2 hrs.

RESULTS

Cerebellar cortex (Figs. 1 and 2) — The molecular layer — At
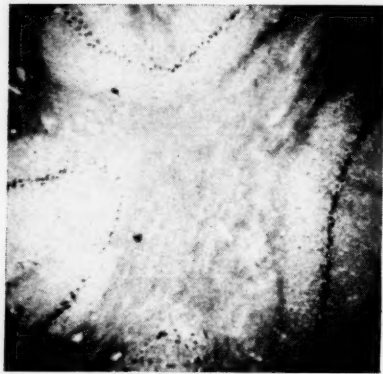
Fig. 1.   Fig. 2.

Figs. 1 and 2. — Rat cerebellum. Strong acid phosphatase activity in the Purkinje cells and in the white matter. 35 ×.
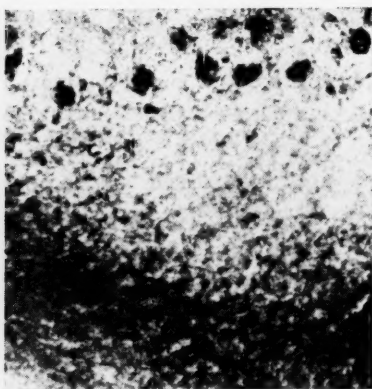
Fig. 3. — Rat cerebellum. Acid phosphatase activity in the Purkinje cells, in the granular layer and in the white matter. 320 ×.

Fig. 4. — Rat cerebellum. Strong acid phosphatase activity in the Purkinje cells and their axons. 800 ×.

a comparison of the activity in the region of the cortical layer with that in other tissue components it is found to be least of all here in the molecular layer. The dendrites of Purkinje's cells are not visible, nor does the method make any different cell types visible, although the activity is slightly different in different places. The Purkinje cells can be clearly distinguished and a strong activity is observed in them (Figs. 1, 2, 3, 4). The activity varies somewhat from cell to cell but not distinct regularity is observable in this

Fig. 5. — Very strong acid phosphatase activity in the plexus chorioideus of the fourth ventricle. 35 ×.
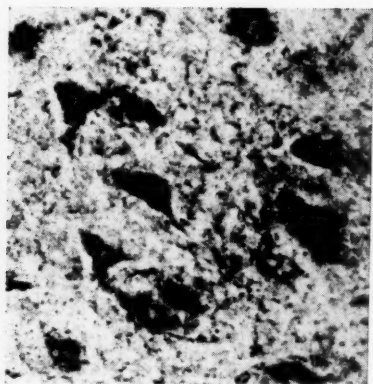
Fig 6.

Fig. 7.

Figs. 6 and 7. — The deep cerebellar nuclei cells. Strong acid phosphatase activity. 630 ×. and 800 ×.

respect. The nucle are clearly visible and in their neighbourhood the strongest activity occurs. The axon of the Purkinje cells is clearly visible in numerous cells over a distance more than the length of the cell. — The granular layer. — (Fig. 3). Here, in the cells a somewhat stronger activity is observable than in the molecular layer. In several places cells with an activity stronger than that in other cells of the corresponding layer can be discerned.

The white matter (Figs. 1, 2, 3). — In the region of the white matter strong activity can be noticed, which falls only slightly short of that observable in the Purkinje cells. In some places one can see how groups of processes depart radially from the white matter towards the Purkinje cells.

The deep cerebellar nuclei and chorioid plexus of the fourth ventricle (Figs. 5, 6, 7). — The deep cerebellar nucleic cells, which are about equal in size to the Purkinje cells although of different shape, resembling as to their structure of the cerebral nucleic cells, are clearly inferior to the Purkinje cells with regard to their acid phosphatase activity. However, the granulation surrounding the nuclei is closer to the Purkinje cells in strength than to the white matter. — Chorioid plexus (Fig. 5). — The strongest activity of all is observed in the chorioid plexus of the fourth ventricle, where the precipitated sulphide of lead indicating the strength of activity stains the said tissue nearly black.

DISCUSSION

It can be noted that the employed method without paraffin embedding and based, instead, on freezing microtomy of the preparation upon fixing in formalin is highly suitable for cerebellar tissue investigations as well. It can clearly be observed in the performed investigation that the acid phosphatase activity is very strong in tissues such as the chorioid plexus; it is obviously due in this case to the secretory function of the plexus. The physiological role of acid phosphatase is still obscure. However, there is evidence that this enzyme participates in the protein synthesis. At comparison of the cells in the various parts of the cerebellum with regard to acid phosphatase activity it is found to be strongest in the Purkinje cells but still inferior in them to that in the chorioid plexus. Obviously the physiological functions in these cells are more lively than in other cells on account of their purpose.

SUMMARY

Acid phosphatase activity in the different parts of the rat's cerebellum has been studied. The strong activity in the chorioid plexus of the fourth ventricle has been compared to that in the various tissues of the cerebellum and the observation has been made that the strongest activity was to be noted in the Purkinje cells and in the deep cerebellar nucleic cells. The activity was third strongest, as observed with the method employed, in the white matter.

REFERENCES

1. COHN, P., and RICHTER, J.: J. Neurochem. 1957:1:166.
2. ERÄNKÖ, O.: Acta physiol. Scandinav. 1951:24:1.
3. ERÄNKÖ, O.: Ann. med. exper. et biol. Fenn. 1951:29:287.
4. LAVELLE, A., LIU, C-N., and LAVELLE, F. W.: Anat. Rec. 1953:115:400.
5. LEDUC, E. H., and WISLOCKI, G. B.: J. Comp. Neur. 1952:97:255.
6. RUTENBERG, A. M., and SELIGMAN, A. M.: J. Histochem. and Cytochem. 1955:3:455.
7. SMITH, W. K., MIALE, J., and FELDMAN, A.: Anat. Rec. 1954:118:409.

FROM THE DEPARTMENT OF ANATOMY, UNIVERSITY OF HELSINKI, AND THE
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NEUROSECRETORY RESPONSE TO EXPERIMENTAL
POLYURIA

by

E. KIVALO and H. ARKO

Gilman and Goodman (4, 5) were the first to show that anti-
diuretic hormone is formed in greater amounts under water depri-
vation; this observation has later been confirmed repeatedly in
dehydration experiments of varying kind. The said authors sug-
gested that the neurohypophysis may respond to the need for water
conservation by secreting increased amounts of antidiuretic hor-
mone. As has been stated by Verney (14, 15), the urine volume is
continually controlled by pituitary secretion, this secretion in turn
being governed by nervous stimuli and osmotic changes in the
blood. Since the antidiuretic hormone strives to maintain a balance
in water economy under water deprivation in accordance with
the reduced supply of liquid, the thought suggests itself that also
at an attempt to disrupt the water balance in the same direction
by artificially induced increased diuresis the organism might strive
to mobilise antidiuretic hormone.

The morphological equivalent of antidiuretic hormone, similarly
as of the other so-called posterior pituitary hormones, which are
probably formed in the hypothalamus, is considered to be the
neurosecretory material observable by histological means in the
hypothalamic-hypophysial system (7, 12). This material has in-
deed been shown to decrease in amount, for instance, at dehydration
experiments (12, 3). The purpose of the present work was to find

out whether also during experimentally increased diuresis such signs can be detected in the neurosecretory material which would give indication of increased production or secretion of antidiuretic hormone.

MATERIAL AND METHODS

28 albino rats of varying ages (weight 120—240 g) and of both sexes, as well as 48 white male mice of the average weight of 20 g were used as experimental animals. The animals thus totalled 76, 44 of them being used for the experiments and 32 serving as controls.

Dehydration. — In order to observe ourselves the neurosecretory response at dehydration, we kept 11 rats on their usual solid mixed food diet but without water. The rats were killed after 2, 4, 6 and 8 days.

Polyuria. — Increased diuresis was caused with a carbonic anhydrase inhibitor, 2-acetylamino-1, 3, 4-thiadiazole-5-sulfonamide (Diamox)[1] which has obviously a purely renal diuretic effect. At least no statements whats-over occur in the literature which would indicate that it might have an effect on the antidiuretic hormone. The rats were given this substance subcutaneously, usually as one injection, a few rats as two injections per day. The dosage varying between 40—160 mg per 100 g and day. The control animals were injected with the same quantity of destilled water. This dosage was maintained for a time varying from a few hours to seven days. The animals were kept on their usual diet and they could take water *ad libitum*. In part of the test and control animals the daily amount of urine and the water intake were followed and it was found that polyuria and polydipsia started rather immediately in the test animals and continued in comparatively unchanged strength throughout the time of administration, i.e., seven days. The mice were given Diamox *per os* during seven days, the dosis varying between 12.5—250 mg per 100 g and day.

The animals were killed swiftly by a blow on the head or by decapitation. The hypophysis was prepared out with a needle and fixed in Bouin's solution for about 24 hours. The organs were dehydrated and embedded in paraffin in the usual manner. They were cut in part with the series microtome and in part with the common microtome at 6 micra. The cuts were stained either with Gomori's chrome-alum-hematoxylin method (2) or with aldehyde-fuchsin (6) upon potassium permanganate oxidation. From three rats which had been given Diamox during seven days also the hypothalamus was removed, as well as from three control rats and they were treated in the same manner as the hypophyses and stained with aldehyde-fuchsin. The adrenals of the last-mentioned test animals were studied with the aid of van Gieson's staining method, as well as the kidneys of one rat which developed hematuria during the test. The best attempt was made to avoid subjective influence at the assessment of the amount of neurosecretory material in the microscopic study.

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[1] Kindly presented by Lederle Laboratories Division.

RESULTS

General Observations. — The subcutaneous Diamox injections seemed to cause intense pain and necroses in the skin; consequently the test rats were frightened and more difficult to manage than the controls which were given solvens only. In the microscopic examination of the adrenal of three test rats performed for this reason no certain pathologic changes could be noticed with van Gieson's staining except in one case where an extensive, rather recent hemorrhage could be seen in the fascicular zone. One rat developed hematuria during three days of treatment. The origin was not visible in the urine passages, and the kidneys appeared normal at microscopic investigation.

Dehydration. — Two days' water deprivation did not seem to cause any diminution of the neurosecretory substance in the rat's neurohypophysis. Only when the dehydration had been continued for six or eight days, a distinct depletion could be observed, the posterior pituitary of the rats kept without liquid for eight days showing stainable substance in a rather scanty amount. The first-mentioned staining method of Gomori was used in this instance.

Diamox-polyuria. — The diuresis of the rats which had been administered Diamox was 2—3 times that of the controls. The water intake of the test animals, too, was greater in about the same proportion. At the microscopic study neither one of the two staining methods employed revealed any difference in the quantity or distribution of the neurosecretory material as compared with the control series. Similarly, no differences could be seen with regard to the aldehyde-fuchsin-positive material in the supraoptic or paraventricular nuclei of the investigated hypothalami as compared to the controls. The neurohypophyses of the mice administered Diamox *per os*, too, appeared normal in regard to the »Gomori substance». No material stainable with the methods employed in this work was observed within the blood vessels in any test or control animal group.

DISCUSSION

The decrease of neurosecretory substance in the posterior pituitary of the rats dehydrated by withdrawal of their drinking water conform to the observations presented before (12, 3) and it can

probably be considered an indication of increased secretion of antidiuretic hormone which would be caused by the change in the hypertonic direction of the osmotic pressure due to water depletion (10). It would seem that the potential contribution of the adrenal gland to this mechanism has not been sufficiently studied.

In the literature dealing with the mutual relations of the hypothalamic-neurohypophysial system and the kidneys hardly any such experimental studies of polyuria can be found in which the first-mentioned system would have remained intact. Moreover, the results of the existing investigations of this kind are not in agreement with each other. For instance, Barrnett (3) states that he has seen both increase and decrease of the neurosecretory substance in the infundibular process of the rat at polydipsia induced by means of exclusive milk-water mixture diet. Our observation that two to threefold increase of diuresis by means of Diamox does not seem to affect the neurosecretory substance, nor probably in consequence the antidiuretic hormone, seems to indicate that mere polyuria as such is not an adequate physiological stimulus for the secretion of antidiuretic hormone but that rather, in according with Verney's (15) classical theory, a change of the osmotic pressure would be required. Such a change could certainly not occur in our experimental animals since they had free access to water and did indeed consume water in an amount corresponding to their diuresis. The loss of electrolytes, too, is undoubtedly without any influence in a test of such short duration (9).

As to a method of investigation of this kind, certain prerequisites have to be considered as satisfied before any conclusions of the kind presented in the foregoing can be made. For the first, one has to assume that the neurosecretory substance and antidiuretic hormone indeed show parallel behaviour. Of this there cannot obviously be any great doubt, considering the ample experimental evidence presented in the literature (12). Secondly it is also necessary to assume that the mobilisation of antidiuretic hormone is reflected with sufficient sensitivity in the histologically demonstrable neurosecretory material without recourse to any quantitative method. This is not immediately evident if one considers the minute quantities concerned when a biologically strongly active substance such as the antidiuretic hormone is required in the physiological functions of the organism. The doubt is enhanced by the above-

mentioned observation that the effect of water deprivation does not become clearly visible in the neurosecretory substance before the lapse of a few days, although it has been established by bioassay that antidiuretic hormone is profusely discharged into the urine already at an early stage of deprivation (5). Furthermore, it has been suggested that the neurosecretory material would merely represent a certain kind of »carrier substance» in similarity to the thyreoid colloid, from which the active principle, in this case the antidiuretic hormone, would pass into the circulation as needed (7), a process of this kind would probably not be readily evident in the histological picture. This distinction of hormones and carriers is disputed by Rothballer (11) who, at the same time, is of the opinion that the neurosecretory substance reflects very sensitively the secretion of posterior pituitary hormones: Even slight unpleasant stimuli (such as pricking with a needle) would cause a reduction of the stainable material, corresponding to the release of antidiuretic hormone observed by means of bioassay, for instance, upon emotional stimuli (15). However, we could not confirm this observation histologically in spite of the fact that the subcutaneous administration of Diamox to rats seemed to constitute a considerable non-specific stress. We shall return to this question in another connection (8).

As regards, on the other hand, the contribution to the functions of the central nervous system of the carbonic anhydrase, which is inhibited by the Diamox injections used as a diuretic in our experiments, hardly anything is known on this subject (13), although the correlation between the quantitative occurence of carbonic anhydrase and the functional development of the brain has been known for a comparatively long time already (1). The circumstance that the neurosecretory substance retained the same strength as in the control material even in the posterior pituitary of rats given Diamox in massive doses for one week, and similarly in those hypothalami which have been investigated, seems to indicate that carbonic anhydrase inhibition, at least by this means, would not affect the neurosecretory activity of the hypothalamic nuclei (the supraoptic and paraventricular nuclei).

SUMMARY

The neurosecretory material of the hypothalamic-hypophysial system, which is considered the morphological equivalent, among others, of the antidiuretic hormone, was investigated by histological methods in white rats and white mice under water deprivation and experimental polyuria. Lack of drinking water maintained for several days caused reduction of the neurosecretory material of the posterior pituitary, obviously as a sign of increased mobilisation of antidiuretic hormone. On the other hand, diuresis increased to 2—3 times its normal amount by means of a carbonic anhydrase inhibitor and corresponding polydipsia did not seem to affect the neurosecretory material and thus probably not the antidiuretic hormone either. It is considered that the results may support the conception of osmotic regulation of the antidiuretic hormone. On the other hand the results seem to indicate that carbonic anhydrase inhibition did not reduce the neurosecretory activity of the hypothalamus. The relations between the histological neurosecretory material and the antidiuretic hormone are briefly discussed.

REFERENCES

1. ASHBY, W.: J. Biol. Chem. 1944:*152*:235.
2. BARGMANN, W.: Ztschr. Zellforsch. mikr. Anat. 1949:*34*:610.
3. BARRNETT, R. J.: Endocrinology 1954:*55*:484.
4. GILMAN, A., and L. S. GOODMAN: Science 1936:*84*:24.
5. GILMAN, A., and L. S. GOODMAN: J. Physiol. 1937:*90*:113.
6. HALMI, N., and J. DAVIES: J. Histochem. and Cytochem. 1953:*1*:447.
7. HILD, W., and G. ZETLER: Ztschr. exper. med. 1953:*120*:236.
8. KIVALO, E., and H. ARKO: Ann. med. exper. et biol. Fenn. 1957, in press.
9. MAREN, T. H., B. C. WADSWORTH, E. K. YALE, and L. G. ALONSO: Bull. Johns Hopkins Hosp. 1954:*95*:277.
10. PETERS, J. P. in G. D. DUNCAN: Diseases of Metabolism, 2nd ed. 1947: 271, W. B. Saunders, Philadelphia.
11. ROTHBALLER, A. B.: Acta neuroveget. 1956:*13*:179.
12. SCHARRER, E., and B. SCHARRER: Neurosekretion, in Handbuch d. mikr. Anatomie 1954:*VI*, *5*:953, Springer, Berlin.
13. SPERRY, W. M. in ELLIOTT, K. A. C., I. H. PAGE, and J. H. QUASTEL: Neurochemistry 1955:252, Charles C. Thomas, Springfield, Illinois.
14. VERNEY, E. B.: Lancet 1929:*1*:539.
15. VERNEY, E. B.: Proc. Roy. Soc. B 1947:*135*:25.

THE EFFECT OF NORADRENALINE AND ACETYLCHOLINE ON WATER DIURESIS AND NEUROSECRETORY SUBSTANCE OF THE RAT

by

E. KIVALO and H. ARKO

There are numerous reports on the experimental effects of adrenal medullary hormones upon renal function. The opinions on the mechanism of these effects are strikingly divided, dependent on the test animals, the dosage of hormones and the experimental conditions. Generally, the action of these hormones is attributed to their effect on blood pressure and/or kidney vasculature (2). However, some evidence derived from diuresis experiments strongly suggests that this action is due, in part at least, to their influence upon the hypothalamo-hypophysial system (3, 5), namely that the antidiuretic hormone would play a role in the phenomena observed after administration of medullary hormones.

On the other hand, it has been shown that the effect of acetylcholine upon the renal excretion of water is due to the release of antidiuretic hormone from the hypothalamo-hypophysial system (13, 1, 2).

It has been suggested in recent years that the antidiuretic hormone is an integral part of the neurosecretory substance which is found histologically in the supraoptic and paraventricular nuclei as well as in the posterior pituitary (14). According to the results of some experiments the antidiuretic hormone is effectually reflected in the occurence of the selectively stainable neurosecretory substance especially in the posterior pituitary (8, 11, 14). The

purpose of the present work was to study the effect of noradrenaline and acetylcholine on water diuresis and, besides, their influence upon the neurosecretory substance in the posterior pituitary of the rat.

MATERIAL AND METHODS

In this work 34 albino rats of both sexes were used. Their weight varied in the range 105—250 g. Eleven rats were given 1-noradrenaline bitartrate subcutaneously in a dosis of 1 mg per kg, 11 other rats were administered acetylcholine chloride subcutaneously, 50 mg per kg, and the remaining 12 control animals were injected with solvens alone. The noradrenaline diuresis was determined with three male rats of average weight 240 g, and with a similar control group. The corresponding determination in the acetylcholine group was made with three male rats of 125 g average weight, which was also the average weight of the control group. Hydration was performed generally according to Ham and Landis (7) as follows: The animals were given water *ad libitum* in their cage up to the commencement of the test. The food had been given on the previous day. At first, the »equilibrium dosis» of water, *i.e.*, $2\frac{1}{2}\%$ of the body weight, was given with the stomach tube and the animals were then placed into metabolism cages in groups of three individuals. After two hours the hydration dosis equalling 5% of the body weight was administered with the stomach tube and the experimental substance, or mere solvens, was injected at the same time. From this time onwards the urine was collected and measured at intervals of 30 minutes during 2—3 hours.

The animals were killed by a blow against the table-edge, 10, 30, 120 and 180 minutes after the administration of substance or solvens. The calvaria was detached, the brain was removed and the hypophyses were extracted with a needle, whereupon they were fixed in Bouin's solution for about 20 hours, dehydrated and embedded in paraffin. The hypophyses were processed to cuts at 5 micra in the frontal plane. They were stained with Gomori's aldehyde-fuchsin method, using potassium permanganate oxydation (6).

RESULTS

Diuresis. — The diuresis caused by *noradrenaline* is seen from Fig. 1. The diuresis starts earlier and continues longer than in the control group. Theoretically 50% excretion of water corresponds to 2½ ml urine per 100 g. Taking from the curves the time for this excretion, it is found to be 74 minutes with the control animals
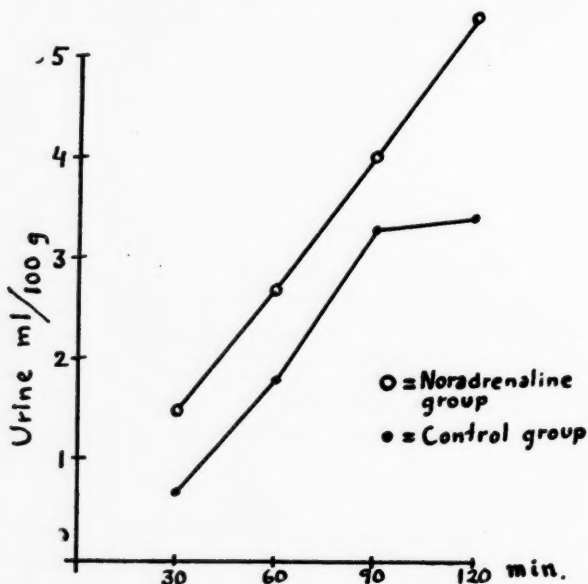
Fig. 1. — A diagram showing the diuresis caused by noradrenaline in the rat.

and 55 minutes with the noradrenaline group. The antidiuresis due to *acetylcholine* is shown in Fig. 2. Actually, here, a delay of diuresis by about one hour has occurred. 50% excretion requires about 109 minutes with the animals under acetylcholine influence, as against 54 minutes in the control group. The animals seemed to endure well both the substances and the manipulation. In the acetylcholine group a conspicuous side effect was bloody dacryorrhea.

Histological Examinations. — The amount of aldehyde-fuchsin-positive material in the posterior pituitary was estimated with the aid of an ordinary light microscope at about $100 \times$ magnification, at which the entire posterior pituitary is visible in the field
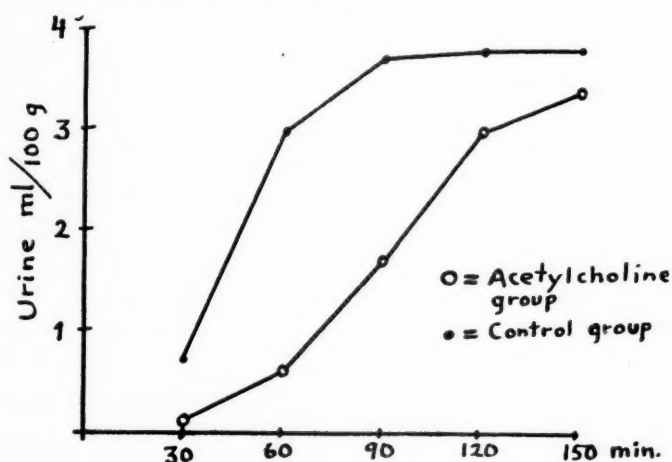
Fig. 2. — A diagram showing the antidiuresis caused by acetylcholine in the rat.

of vision. The samples were classified without knowledge of their identity into three groups according to the abundance of stainable material. This procedure revealed no significant differences between the noradrenaline and acetylcholine groups, nor did hydration, sex or weight appear to have any influence in this respect. Attention was also paid to the width of the blood vessels and the occurence of neurosecretory material in them, but stainable substance was not once observed within the lumen; the relative vasodilatation, which was observed quite frequently, showed no distinct correlation with the treatment of the experimental animals.

DISCUSSION

In hydrated rats noradrenaline caused considerable diuresis, as has been observed earlier, too (10, 4). The increase seemed to be based on the circumstance that the diuresis commenced earlier and persisted longer than in the control group. However, the time difference relating to 50% excretion was not as great in our experiment as has been reported (4). It is difficult to explain why noradrenaline causes diuresis in the rat but antidiuresis, for instance, in the dog (2, 5, 3). The difference can hardly be due to the dosage since smaller doses, too, have a diuretic effect in rats (10).

Acetylcholine caused highly distinct antidiuresis in rats for

about one hour. Thus, diuresis produced by means of water strain, which continued at great strength for about $1\frac{1}{2}$ hours in the control groups, was delayed in the acetylcholine group and did not begin to subside until after about $2\frac{1}{2}$ hours. Such prolonged antidiuresis is indeed resemblant of the effect of the antidiuretic substance (15) and earlier research suggests strongly that the inhibition of urine flow caused by acetylcholine is due to the release of antidiuretic hormone from the posterior pituitary (12, 13,1).

At the histological examination we could not observe any change in the amount of neurosecretory substance in the posterior pituitary or in its distribution, regardless of the varying time of action (10—180 minutes) of the administered substances. Water hydration as such did not either seem to have any effect whatsoever.

On the strength of our results we cannot furnish any additional elucidation to the question whether noradrenaline affects the antidiuretic substance. However, although acetylcholine obviously produces discharge of antidiuretic hormone (as well as of oxytocin), nothing can be seen in the posterior pituitary in this case either. This rather suggests that temporary mobilisation of the antidiuretic hormone is not very readily evident at the histogical examination, at least not without recourse to a more accurate quantitative method. This is plausible enough, since evidently very small quantities of the substance are involved. The reason may also be that in the neurosecretory substance the histologically stainable part probably merely represents a carrier (9), which may not disappear simultaneously with the active principles.

SUMMARY

The effect of one single subcutaneous noradrenaline and acetylcholine injection, respectively, upon the diuresis of albino rats has been investigated and simultaneously that upon the neurosecretory substance of their posterior pituitary, which is considered to contain the antidiuretic hormone. In hydrated rats noradrenaline caused slight diuresis, and acetylcholine caused marked inhibition of urine flow for about one hour. Noradrenaline and acetylcholine, as well as hydration as such, did not produce within 10—180 minutes any changes in the aldehyde-fuchsin-positive neurosecretory material of the posterior pituitary.

REFERENCES

1. ABRAHAMS, V. C., AND M. PICKFORD: J. Physiol. 1954:*126:*329.
2. BLAKE, W. D.: Am. J. Physiol. 1955:*181:*399.
3. DEARBORN, E. H., and L. LASAGNA: J. Pharmacol. 1952:*106:*122.
4. DRILL, V. A., and W. R. BRISTOL: Endocrinology 1951:*49:*589.
5. ERÄNKÖ, O., M. J. KARVONEN, A. LAAMANEN, and M. E. PITKÄNEN: Acta pharmacol. et toxicol. 1953:*9:*345.
6. HALMI, N. S., and J. DAVIES: J. Histochem. and Cytochem. 1953:*1:*447.
7. HAM, G. C., and E. M. LANDIS: J. Clin. Invest. 1942:*21:*455.
8. HILD, W.: Virchows Arch.f.path. Anat. 1951:*319:*526.
9. HILD, W., and G. ZETLER: Ztschr.exper. Med. 1953:*120:*236.
10. HORRES, A. D., W. J. EVERSOLE, and M. H. ROCK: Proc. Soc. Exp. Biol. Med. 1950:*75:*58.
11. ORTMANN, R.: Ztschr. Zellforsch. mikr. Anat. 1951:*36:*92.
12. PICKFORD, M.: J. Physiol. 1939:*95:*226.
13. PICKFORD, M., and J. A. WATT: J. Physiol. 1951:*114:*333.
14. SCHARRER, E., and B. SCHARRER: Neurosekretion, in Handbuch d. mikr. Anatomie 1954:*VI*, *5:*953, Springer, Berlin.
15. VERNEY, E. B.: Proc. Roy. Soc. B 1947:*135:*25.

NEUROSECRETORY SUBSTANCE IN THE NEURO-
HYPOPHYSIS OF THE RAT

INFLUENCE OF SOME ANTIDIURETIC DRUGS

by

H. ARKO, E. KIVALO and U. K. RINNE

It is known that the release of neurohypophysial hormones can be stimulated by means of a number of drugs. Such drugs, the anti-diuretic mechanism of action of which has been clarified with com-parative reliability and seems to have respect to the release of antidiuretic hormone from the posterior pituitary, are:acetylcholine (19), adenosine triphosphate (5), ether (10), ferritin (1), morphine (2), nicotine (4), pentobarbitone and phenobarbitone (3), and yohim-bine (8).

In our investigations concerning the relation of antidiuretic hormone to the histologically demonstrable hypothalamo-hypo-physial neurosecretory substance, two of the present authors (17) have already studied, by histological means, the effect of acetyl-choline upon the neurohypophysial neurosecretory substance. As the result was not fully in accordance with expectations, we decided to continue the experiments also with the other above-mentioned drugs which possess antidiuretic effect.

MATERIAL AND METHODS

The homogeneous experimental consisted of 46 female albino rats, all weighing approximately 250 g, which were given food and water *ad libitum* up to the commencement of tests. The control

group comprised 16 rats; four of them were kept as untreated controls, whereas eight animals were given subcutaneously once 0.5 cc. water and four animals were given the same water quantity three times at half-hour intervals.

The following substances were used in the experiment: Adenosine triphosphate (»Adenosine-5-triphosphoric acid», L. Light & Co.) in doses of 0.01 g per kg of weight, morphine (»Morphin», Orion) 0.005 g per kg, phenobarbitone (»Luminal», Bayer) 0.06 g per kg. nicotine (»Nicotine Hydrogen Tartrate», British Drug House) 0.005 g per kg, yohimbine (»Yohimbin, hydrochlor.», Orion) 0.02 g per kg. Each one of the drugs was administered subcutaneously to two rats as one single injection and to two rats three times at half-hour intervals. All animals were sacrificed 30 minutes after the last injection. The phenobarbitone group constitutes an exception in that two animals received a single injection and two rats were give two injections at an interval of one hour, for the reason that they would have slept with more frequent administration of this drug.

In addition, two animals were killed in less than five minutes with ether and two further rats after ether administration prolonged for one hour (»Aether ad Narcosin», Orion).

In order to control the effect of phenobarbitone, it was further- more given in the above-mentioned dosis to four rats, injecting at the same time two rats with pentobarbitone (»Nembutal», Abbot) in doses of 0.025 g per kg of weight; the inclusion of this latter drug in the tests had not been planned originally since it has been found to possess an antidiuretic effect inferior to that of pheno- barbitone (3). Ferritin was not included in the experiments on account of difficulties in obtaining this drug, owing to its high price. The animals were killed by a hard blow against the table- edge, and the hypophyses were removed immediately. After 24 hours fixing in Bouin's solution the organs were treated in the usual manner and embedded in paraffin. Cuts of 4 micra were stained by Gomori's aldehyde-fuchsin method, preceded by $KMnO_4$ oxida- tion (12).

RESULTS

The animals endured the employed dosage satisfactorily, although some drugs had an evident effect upon the behaviour of

the rats: for instance, drowsiness with the barbiturates, dragging of the hind legs or slight convulsion with nicotine, etc. In our opinion the dosages employed in the test were suitable for an acute experiment and it can be assumed that they had also sufficient stimulating effect upon the antidiuretic hormone, according to the literature references cited in the introduction.

At the performed microscopic investigation on the whole no definite signs of reduction of the neurosecretory substance could be observed. Only in a few rats which had received one phenobarbitone injection ½ hour prior to extermination could depletion of the aldehyde-fuchsin-positive substance be noted, but this was not the case in all such animals, nor in those which had been subjected to prolonged treatment. The administration of ether during one hour, too, has possibly reduced the stainable substance in the posterior pituitary but not the ether treatment of short duration. Particular attention was paid to the blood vessels of the posterior pituitary, but stainable substance was not seen even once within their lumen in any group of animals.

DISCUSSION

The slight depletion of the neurosecretory substance observed after ether anaesthesia prolonged for one hour might be attributed to the known stimulating effect of ether upon the antidiuretic hormone (10,7). However one would then expect the same effect with the other substances employed in the test, unless the effect of ether is particularly due to its stressor property as an unpleasant stimulus (20) which, yet, we do not believe to have any significant influence in this instance (16). Certainly, judging from their behavior, e.g. the rats treated with nicotine can hardly have felt any better, but no depletion occured in the case of these animals. Possibly ether acts by its purely chemical properties, e.g., as a solvent, upon the stainability of the neurosecretory substance. Rapid ether induction having a fatal result within a few minutes was not found to deplete the aldehyde-fuchsin-positive substance in the posterior pituitary.

In some rats which had been given phenobarbitone once, depletion of the neurosecretory substance was noted, but not in all

of them. Peculiarly enough the animals administered two doses at an interval of one hour, again, did not show any difference from the controls. The reason of this discrepancy is not clarified. We think that it could be arteficial.

The circumstance that with the above-mentioned expections we could not as a rule observe any depletion of the neurosecretory substance in the posterior pituitary nor any transfer of this substance into the blood vessels although the drugs administered to the rats cause, according to earlier investigation, mobilisation of antidiuretic hormone from the hypothalamo-hypophysial system into the blood, supports in our opinion the conception of Hild and Zetler (14), *i.e.*, that the stainable substance constitutes only a carrier molecule, not the pharmacologically active elements. This distinction is disputed by Rothballer (20). Possibly, nothing happens to the carrier at the moment of release of neurohypophysial hormones and it disappears, or at least ceases to be stainable, only at a considerably later time. Some other observations, too, which have also confirmed by ourselves (15), can be explained as being support of this contention of ours, namely, the fact that prolonged water deprivation, at which antidiuretic hormone is secreted in greater than normal amounts already at an early stage (11), causes depletion of the neurosecretory substance in the posterior pituitary (e.g. 18, 13), whereas less prolonged lack of drinking water or some other temporary stimulus of antidiuretic hormone (nicotine injection) does not reduce the quantity of this material (9). Furthermore, it has been stated that no distinct parallelity exists between the neurosecretory substance and oxytocin, which is also considered as belonging to the same (6). In our opinion, thus there is evidence to the effect that a certain distinction between the carrier substance and the posterior pituitary hormones is required.

SUMMARY

The effect upon the neurosecretory substance of the rats posterior pituitary of certain substances acceredited with the ability to stimulate the secretion of antidiuretic hormone (adenosine triphosphate, ether, morphine, nicotine, pentobarbitone, phenobarbitone, yohimbine) has been investigated with the aid of aldehyde-

fuchsin stainig. With the depletion of the neurosecretory substance occasionally observed at the administration of phenobarbitone and ether as exceptions, no signs of release of antidiuretic hormone could be observed in the histological material. The relation between antidiuretic hormone and the histological neurosecretory substance is briefly discussed.

REFERENCES

1. Baez, S., A. Masur, and E. Shorr: Am. J. Physiol. 1952:*169:*123.
2. de Bodo, R. C.: J. Pharmacol. 1944:*82:*74.
3. de Bodo, R. C., and K. F. Prescott: J. Pharmacol. 1945:*85:*222.
4. Burn, J. H., L. H. Truelove, and J. Burn: Brit. med. J. 1945:*1:*403.
5. Dexter, D., H. B. Stoner, and H. N. Green: J. Endocrinol. 1954:*11:* 142.
6. Drager, G. A., and E. G. Rennels: Anat. Rec. 1955:*121:*287.
7. Eversole, W. J., and F. A. Giere: Feder. Proc. 1954:*13:*42.
8. Fugo, N. W.: Endocrinology 1944:*34:*143.
9. Gaunt, R., C. W. Lloyd, and J. J. Chart: in Heller, H.: The Neurohypophysis. 1957 Butterworth's Sc. Publ., London, p. 242.
10. Ginsburg, M., and H. Heller: J. Endocrinol. 1953:*9:*274.
11. Gilman, A., and L. S. Goodman: Science 1936:*84:*24.
12. Halmi, N. S., and J. Davies: J. Histochem. and Cytochem. 1953:*1:*447.
13. Hild, W.: Virchows Arch. f. path. Anat. 1951:*319:*526.
14. Hild, W., and G. Zetler: Ztschr. f. ges. exper. Med. 1953:*120:*236.
15. Kivalo, E., and H. Arko: Ann. Med. Exper. et Biol. Fenn. 1957 in press.
16. Kivalo, E., and H. Arko: Ann. Med. Exper. et Biol. Fenn. 1957 in press.
17. Kivalo, E., and H. Arko: Ann. Med. Exper. et Biol. Fenn. 1957 in press.
18. Ortmann, R.: Z. Zellforsch. 1951:*36:*92.
19. Pickford, M.: J. Physiol. 1939:*95:*226.
20. Rothballer, A. B.: Acta neurovegetativa 1956:*13:*179.

ÜBER DEN ZUSAMMENHANG ZWISCHEN BLUTEIWEISS-KÖRPER UND KOLLOIDOSMOTISCHEM DRUCK

by

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Der kolloidosmotische Druck soll zunächst durch die Serumproteine bedingt sein. Neben dem Albumin und den Globulinen ist der Anteil des Fibrogens in der Praxis ohne Bedeutung. Der kolloidosmotische Druck ist praktisch genommen im Blut, im Plasma und im Serum gleich (1). Der Anteil der anderen Kolloide, z.B. der Lipoide, wird für so gering angesehen, dass er ausser acht gelassen werden kann (20, 21). Es sind aber auch andere Auffassungen vorgebracht worden (4). Es ist möglich, dass die Lipoproteinuntersuchungen neue Gesichtspunkte darbieten werden.

Da die direkte Messung des kolloidosmotischen Druckes immer mit praktischen Schwierigkeiten verbunden gewesen ist, hat man versucht, mit Hilfe von indirekten Bestimmungsmethoden zum Ziel zu kommen. Dieselben basieren auf Eiweissanalysen, bei denen versucht wird, den Anteil der verschiedenen Eiweisskomponenten an der Entstehung des kolloidosmotischen Druckes zu bestimmen. Die grosse molare Konzentration des Albumins im Vergleich zu den anderen Proteinen bedeutet, dass sein Anteil den anderen Proteinen gegenüber am beträchtlichsten ist. Die Bedeutung des Albumins wird noch dadurch erhöht, dass sein isoelektrischer Punkt weiter vom pH des Blutes entfernt ist als bei den Globulinen, so dass es im Blut ein stärker ionisierter Kolloidelektrolyt ist als die Globuline.

Es sind verschiedene Formeln veröffentlicht worden, nach

denen sich der kolloidosmotische Druck errechnen lässt, wenn die Menge des Gesamteiweisses, des Albumins und der Globuline bekannt ist. Die Formeln enthalten ausserdem Konstanten, die von der Eiweisskonzentration der zu untersuchenden Lösung abhängig sein können. Ott (19) hat festgestellt, dass der kolloidosmotische Serumdruck auf Grund der Elektrophoreseanalyse mit einer Genauigkeit von $\pm$ 6.7% berechnet werden kann.

Obschon mit Hilfe von Messkontrollen festgestellt werden konnte, dass sich der kolloidosmotische Druck in vielen Fällen durch Untersuchung des Eiweissgehaltes befriedigend ermitteln lässt, sind doch auch Zweifel an der Zuverlässigkeit derartiger Berechnungen vorgebracht worden. So hat man z.B. vermutet, dass *der kolloidosmotische Druck irgendwie auch vom Zustand der Eiweisskörper abhängig sein könnte, was in den Proteinanalysen überhaupt nicht zum Vorschein kommt* (3, 8, 10, 12, 14, 15, 22, 24, 25, 29). Hiermit könnten die klinischen Beobachtungen übereinstimmen, nach denen sich trotz zahlreicher Untersuchungen keine sichere Ödemgrenze nachweisen lässt, die mit niedrigen Serumproteinen verknüpft wäre, obschon die Proteine bei der Entstehung des Ödems nur ein Teilfaktor sind.

Es ist festgestellt worden, dass bei Kaninchen die Veränderungen in den Serumproteinen und im kolloidosmotischen Druck nicht immer parallel laufen (5, 6, 11, 17, 28). — Man hat beobachtet, dass in Serumverdünnungen der kolloidosmotische Druck schneller absinkt als die Eiweisskonzentration (25). Bei niedrigen Proteinkonzentrationen wurde festgestellt, dass der kolloidosmotische Druck in vitro sich insbesondere in »pathologischen Seren» schnell verändert (27).

Insbesondere die für den klinischen Gebrauch geeigneten Formeln sollten in unternormalen Eiweisskonzentrationen zuverlässig sein. In der Praxis sind wenigstens bis auf weiteres die pathologisch niedrigen Werte des kolloidosmotischen Druckes wichtiger als die erhöhten.

Mit Hilfe der elektrophoretischen Eiweissanalyse ist in der vorliegenden Arbeit die Eignung einiger Formeln zur Bestimmung des kolloidosmotischen Druckes ausprobiert worden, indem die auf Grund der Eiweissanalyse errechneten Werte des kolloidosmotischen Drucks mit den durch Messung erhaltenen verglichen wurden.

UNTERSUCHUNGEN

Es wurden 27 Seren untersucht. Die Proben verteilten sich folgendermassen: Nr. 1, 3, 4, 5, 6 Herzinsuffizienz, 2, 7 Perniziöse Anämie, 8 Chronische Nephritis, 26, 27 Multiple Myelome, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 Gesunde.

Von den Proben wurden die Gesamtproteine mit $CuSO_4$, nach Kjelldahl sowie mit Hilfe der Elektrophorese bestimmt, indem die Fraktionen zusammengerechnet wurden. Die Abweichung der aus der Elektrophorese berechneten Gesamtproteine von den mit anderen Methoden erhalten Werten betrug $\pm$ 0.2%. Die Elektrophoreseanalyse wurde mit dem Mikroelektrophoreseapparat von Antweiler (Boskamp AG) durchgeführt, wobei das Albumin sowie die $a_1 a_2 \beta \gamma$ und δ-Globuline bestimmt wurden. Die Analysenergebnisse sind aus der Tabelle 1 ersichtlich.

Der kolloidosmotische Druck einer jeden Probe wurde nach dem Verfahren von Holm-Jensen (9) gemessen. Der Messungsfehler beträgt etwa ±1 cm H_2O. Dieses Verfahren ist später weiter entwickelt worden (18). Die Messungsergebnisse sind in der Tabelle 1 wiedergegeben.

Die gemessenen Werte des kolloidosmotischen Druckes wurden dann mit den errechneten Werten verglichen, welche letzteren durch Einsetzen der von den Eiweissanalysen ergebenen Werten in die Formel erhalten wurden. Die Berechnungen wurden nach folgenden fünf Formeln durchgeführt:

1. $P = C (21.4 + 5.9 A)$. P = kolloidosmotischer Druck, C = Gesamteiweiss, A = Albumin (26).

2. $P = fc (45.2 A + 18.8 G) \dfrac{T}{273}$. P = kolloidosmotischer Druck, fc = von der Eiweisskonzentration abhängige Konstante, A = Alb., G = Globul., T = abs. Temperatur (13).

3. $P = C (21.1 + 5.2 A)$. P = kolloidosmotischer Druck, C = Gesamteiweiss, A = Alb. (7).

4. $3.56 A + G = 0.06 P$. A = Alb., G = Globul., P = kolloidosmotischer Druck (2).

5. $p [v — 0.0182 (q — 1.39)^2 — 0.0415] = \sqrt{q + 0.2} + 2.185$. p = kolloidosmotischer Druck, v = umgekehrter Wert der Eiweisskonzentration, q = Verhältnis zwischen Albumin und Globulinen (16).

TABELLE 1

DIE RESULTATE DER EIWEISSANALYSE UND KOLLOIDDRUCKMESSUNGEN

Nr.	Gesamt eiweiss	alb.	glob.	α_1	α_2	β	γ	δ	alb. / glob.	Kolloid Druck cm H_2O
1.	5.62	1.89	3.73	0.25	0.22	0.58	2.02	0.06	0.51	17.5
2.	4.81	3.39	1.42	0.31	0.06	0.45	0.58	0.04	2.39	17
3.	5.61	2.71	2.90	0.53	0.67	1.04	0.44	0.22	0.93	19
4.	6.01	2.60	3.41	0.69	0.70	0.71	1.12	0.19	0.76	22
5.	6.75	1.90	4.85	0.63	0.66	0.63	2.69	0.24	0.39	31.5
6.	5.85	3.00	2.85	0.60	0.15	0.38	1.63	0.09	1.05	21
7.	5.63	3.56	2.07	0.20	0.15	0.35	1.26	0.11	1.72	26
8.	5.79	3.71	2.08	0.39	0.70	0.21	0.70	0.08	1.78	21
9.	5.74	3.85	1.89	0.58	0.15	0.43	0.64	0.09	2.03	29
10.	6.96	3.48	3.48	0.55	0.73	0.61	1.49	0.10	1.00	22
11.	6.60	4.05	2.56	0.84	0.15	0.54	0.89	0.14	1.58	27
12.	6.69	4.03	2.66	0.59	0.40	1.06	0.52	0.09	1.52	30.5
13.	6.93	3.83	3.10	1.14	0.40	0.28	1.20	0.08	1.23	34
14.	6.72	4.21	2.51	0.72	0.13	0.48	0.99	0.22	1.68	22.5
15.	7.01	3.94	3.07	0.84	0.46	0.82	0.81	0.14	1.28	31.5
16.	6.91	4.29	2.62	0.77	0.82	0.20	0.54	0.29	1.64	25.5
17.	7.18	3.98	3.20	0.32	0.08	0.90	1.69	0.21	1.24	35.5
18.	7.46	3.89	3.57	0.67	0.33	0.93	1.51	0.13	1.09	29
19.	7.01	4.48	2.53	0.82	0.11	0.40	1.10	0.09	1.77	33
20.	7.10	4.59	2.51	0.81	0.36	0.49	0.70	0.15	1.83	28
21.	6.89	4.79	2.10	0.91	0.16	0.18	0.64	0.21	2.28	25.5
22.	6.89	4.52	2.37	1.10	0.11	0.43	0.48	0.25	1.84	26
23.	8.69	3.40	5.29	0.67	0.35	0.56	3.49	0.22	0.64	36
24.	8.04	4.46	3.58	0.93	0.40	1.66	0.82	0.06	1.25	29
25.	7.10	4.30	2.80	0.65	0.45	0.84	0.86	—	1.54	28.5
26.	7.61	4.90	2.71	0.96	0.09	0.58	1.09	0.21	1.81	28.5
27.	8.33	3.92	4.41	0.75	0.13	0.80	2.52	0.21	0.89	24

Die Resultate sind in der Tabelle 2 wiedergegeben.

Eine Zusammenfassung gibt Tabelle 3, worin die Abweichungen der nach den verschiedenen Formeln erhaltenen Werten voneinander und von dem gemessenen Wert des kolloidosmotischen Drucks angegeben sind.

TABELLE 2

DIE KOLLOIDOSMOTISCHEN DRUCKWERTE NACH FÜNF FORMELN ERRECHNET UND
DIE GEMESSENEN WERTE CM H_2O

Nr.	Messungs werte	Formel 1	Formel 2	Formel 3	Formel 4	Formel 5
1.	17.5	18	13	17.5	17.5	25.5
2.	17	20	14.5	22	22.5	25.5
3.	19	21	14.5	19.5	21	19
4.	22	22	15.5	20.5	21	26.5
5.	31.5	22	15.5	21	19.5	33.5
6.	21	23	16	21.5	22.5	20
7.	26	24	16.5	23.5	24.5	30
8.	21	25	17	23.5	25.5	28
9.	29	25	17	23,5	26	29.5
10.	22	29	20	27	28	33
11.	27	30	20.5	28	28.5	32
12.	30.5	30	20.5	28	29	35
13.	34	30.5	21.5	28.5	28	33
14.	22.5	31	21	29	29	34
15.	31.5	31	22	29	28.5	35
16.	25.5	32.5	23	30	30	35
17.	35.5	32.5	22.5	30	29	35
18.	29	33	24	31	28	25
19.	33	33.5	23	31	32.5	40.5
20·	28	34	23.5	31.5	32	26
21.	25.5	34.5	24	32	31.5	35
22.	26	35	23	31	31	36.5
23.	36	36	25.5	33.5	29	49
24.	29	37	27	35.5	32.5	42.5
25.	28.5	37	24	31	30	25
26.	28.5	38	27	35.5	33.5	41.5
27.	24	37	26	34.5	30.5	43.5

TABELLE 3

DIE ABWEICHUNGEN DER NACH FÜNF FORMELN ERRECHNETEN KOLLOIDDRUCK-
WERTE VON DEN GEMESSENEN WERTE CM H_2O

Nr.	Formel 1	Formel 2	Formel 3	Formel 4	Formel 5
1.	+ 1.5	— 4.5	0	0	+ 8
2.	+ 3	— 2.5	+ 5	+ 5.5	+ 8.5
3.	+ 2	— 4.5	+ 0.5	+ 2	0
4.	0	— 6.5	— 1.5	— 1	+ 4.5
5.	— 9.5	—16	—10.5	—12	+ 2
6.	+ 2	— 5	+ 0.5	+ 1.5	— 1
7.	— 2	— 9.5	— 2.5	— 1.5	+ 4
8.	+ 4	— 4	+ 2.5	+ 4.5	+ 7
9.	— 4	—12	— 5.5	— 3	+ 0.5
10.	— 7	— 2	+ 5	+ 6	+11
11.	+ 3	— 6.5	+ 1	+ 1.5	+ 5
12·	— 0.5	—10	— 2.5	— 1.5	+ 4.5
13·	— 3.5	—12.5	— 5.5	— 6	— 1
14·	+ 8.5	— 1.5	+ 6.5	+ 6.5	+11.5
15·	— 0.5	— 9.5	— 2.5	— 3	+ 3.5
16·	+ 7	— 2.5	+ 4.5	+ 4.5	+ 9.5
17·	— 3	—13	— 5.5	— 6.5	— 0.5
18·	+ 4	— 5	+ 2	— 1	— 4
19·	+ 0.5	—10	— 2	— 0.5	+ 7.5
20.	+ 6	— 4.5	+ 3.5	+ 4	— 2
21·	+ 9	— 1.5	+ 6.5	+ 6	+ 9.5
22.	+ 9	— 3	+ 5	+ 5	+10.5
23.	0	—10.5	— 2.5	— 7	+13
24.	+ 8	— 2	+ 6.5	+ 3.5	+13.5
25.	+ 8.5	— 4.5	+ 2.5	+ 1.5	— 3.5
26.	+ 9.5	— 1.5	+ 7	+ 5	+13
27.	+13	+ 2	+10.5	+ 6.5	+19.5

BESPRECHUNG DER ERGEBNISSE

Wie aus Tabelle 1 und 2 hervorgeht, schwankte der gemessene kolloidosmotische Druck unserer Seren zwischen 17 und 35.5 cm H_2O. Die Tabellen 2 und 3 zeigen, dass keine von den angewandten Formeln mit der Messung übereinstimmende Werte ergab. In dieser Hinsicht bestand zwischen den normalen und den pathologischen Seren kein Unterschied. Besonders auffallend ist es, dass die nach den verschiedenen Formeln berechneten Resultate erheblich voneinander abweichen. Am nächsten kamen einander die Ergebnisse der Formeln 3 und 4, sie wichen aber von den gemessenen Werten nach beiden Richtungen hin ab. Mit Ausnahme von einem Falle (Nr. 27) ergab die Formel 2 jedesmal ein kleineres Resultat als der gemessene Wert. Mit der Formel 5 wiederum wurde in 20 von 27 Fällen ein grösserer Wert als beim Messen erhalten. Der in jedem einzelnen Falle für den kolloidosmotischen Druck errechnete kleinste und grösste Wert wichen voneinander mindestens 6.5 cm H_2O (Nr. 3) und höchstens 23.5 cm H_2O (Nr. 23) ab. Dies bedeutet, dass im Zusammenhang mit einer jeden Probe irgendeine Formel einen Fehler von etwa 10% und in vielen Fällen von etwa 50% ergeben hatte.

Die Korrelation zwischen kolloidosmotischem Druck und Albuminkonzentration zeigte sich in den meisten Fällen, aber nicht immer (Nr. 2, 14, 16 und 21). Das Verhältnis zwischen Albumin und den Globulinen scheint keine Bedeutung zu haben. Auch die relativen Mengen der Globulinkomponenten schienen mit dem kolloidosmotischen Druck in keinem Zusammenhang zu stehen. Das Resultat steht mit der Auffassung von Scatchard (23) nicht im Einklang.

Es kann sein, dass in einigen Fällen der Unterschied zwischen den gemessenen und den errechneten Werten auf einem fehlerhaften Messungsergebnis beruht. Es möge jedoch darauf hingewiesen werden, dass immer irgendeine von den fünf angewandten Formeln einen Wert ergeben hatte, der dem Messungsergebnis verhältnismässig nahekam. Die grösste Abweichung zwischen dem Messungsergebnis und dem nächstliegenden berechneten Wert ist 3 cm H_2O (Nr. 22). — Unsere Ergebnisse weisen also darauf hin, dass der auf Grund der Eiweissanalyse errechnete kolloidosmo-

tische Druck weitgehend vom gemessenen Wert abweichen kann
Man sollte somit bestrebt sein, Messungsmethoden zu entwickeln
sofern man den kolloidosmotischen Druck genau ermitteln will.

ZUSAMMENFASSUNG

Es ist der kolloidosmotische Druck von 27 Seren mit unter-
schiedlichem Eiweissgehalt auf zwei verschiedene Weisen unter-
sucht worden, und zwar a) durch Berechnung nach elektrophore-
tischer Eiweissanalyse und b) durch Messung nach der Methode von
Holm-Jensen (9). Die Berechnungen wurden nach fünf verschie-
denen Formeln durchgeführt, und die so erhaltenen Werte wurden
miteinander sowie mit den durch Messung erhaltenen Werten ver-
glichen. Es wurde festgestellt, dass die Werte erheblich voneinander
abwichen, und zwar sowohl bei der Berechnung nach den ver-
schiedenen Formeln als auch im Vergleich zu den durch Messung
erhaltenen Werten. In den meisten Fällen wurde eine Korrelation
zwischen dem Albumin und dem kolloidosmotischen Druck fest-
gestellt, jedoch nicht immer. Das Albumin-Globulin-Verhältnis
oder die verschiedenen Globulinfraktionen schienen bezüglich des
kolloidosmotischen Druckes keine Bedeutung zu haben. Die Resul-
tate scheinen darauf hinzudeuten, dass die direkte Messung des
kolloidosmotischen Druckes zuverlässiger sei als die Berechnung
aus den Proteinkonzentrationen.

LITERATURVERZEICHNIS

1. BENNHOLD, H., KYLIN, E., und RUSZNYAK, S.: Die Eiweisskörper des
   Blutplasmas. — Dresden und Leipzig 1938.
2. BJØRNEBOE, M., BRUN, C., und RAASCHOU, F.: Acta Med. Scandinav.
   1948:*Suppl. 206.*
3. BUTT, H. R., und KEYS, A.: Proc. Staff Meet., Mayo Clin. 1937:*12:*566.
4. CARRIÈRE, G., HURIEZ, C., und LEPERRE, M.: Presse Med. 1936:*44:*225.
5. FUKUHARA, R.: Tohoku J. Exper. Med. 1936:*30:*465.
6. FUKUHARA, R.: Tohoku J. Exper. Med. 1937:*30:*506.
7. GORECZKY, L., und HÉTHELYI, F.: Biochem. Ztschr. 1942:*311:*15.
8. GOVAERTS, P.: Compt. rend. Soc. de biol. 1924:*91:*116.
9. HOLM-JENSEN, I.: Scandinav. J. Clin. & Lab. Investigation 1949:*1:*70.
10. JELKE, H.: Svenska läkartidn. 1953:*41:*2110.
11. KASUGAI, F.: Tohoku J. Exper. Med. 1935:*27:*467.

12. Kessler, R., und Paulsen, F.: Acta med. Scandinav. 1935:*86*:100.
13. Keys, A.: J. Phys. Chem. 1938:*42*:11.
14. Krogh, A., und Nakazawa, F.: Biochem. Ztschr. 1927:*188*:241.
15. Mayrs, E. B.: Quart. J. Med. 1926:*19*:273.
16. Meyer, P.: Science 1951:*113*:279.
17. Miyamoto, T., und Sugimoto, H.: Tohoku J. Exper. Med. 1934:*34*:238.
18. Neidhardt, F. O.: Undersøgelser over Dextrans Indvirkning Paa Serums Kolloidosmotiske Tryk Hos Kaniner. — Aarhus 1955.
19. Ott, H.: Klin. Wchnschr. 1956:*39/40*:1079.
20. Popják, G., und Mc Carthy, E. F.: Biochem. J. 1943:*37*:702.
21. Popják, G., und Mc Carthy, E. F.: Biochem. J. 1946:*40*:789.
22. Reid, E. W.: J. Physiol. 1904:*31*:438.
23. Scatchard, G.: Science 1951:*113*:201.
24. Torbert, H. C., und Cheney, G.: J.A.M.A. 1936:*106*:683.
25. Verney, E. B.: J. Physiol. 1926:*61*:319.
26. Wells, H. S., Youmans, J. B., und Miller, D. G. Jr.: J. Clin. Investigation 1935:*12*:1103.
27. Yanagi, J.: J. Clin. Investigation 1935:*14*:853.
28. Yasuda, H.: Tohoku J. Exper. Med. 1937:*31*:437.
29. Zozaya, J.: J. Physic. Chem. 1938:*42*:657.

FROM THE DEPARTMENT OF PATHOLOGY, SECTION II AND THE EYE HOSPITAL, UNIVERSITY OF HELSINKI

THE SCLERAL DRAINAGE AREA

HISTOLOGICAL STUDIES

by

HARALD TEIR, M. D. and SALME VANNAS, M. D.

It has been fairly often suggested that aqueous outflow is affected by some kind of resistance which has been assumed to occur either in the trabecular meshwork (4, 18) or in the area distal to Schlemm's canal (2², 13).

Our knowledge of the anatomy and morbid anatomy of the region of the eye which is responsible for a possible control of drainage and aqueous outflow from the anterior chamber is limited (8, 2³). The anatomy of the trabecular meshwork has, however, been carefully studied in the last few years by Ashton, Brini and Smith, and by Flocks. Vrabec, moreover, found a metachromatically staining ground substance in the trabecular meshwork.

In the discussion of the aqueous outflow barrier attention has usually been concentrated on the trabecular meshwork. The chief interest in our study was the structures in the anterior part of the sclera and especially peripheral to Schlemm's canal. The purpose of this paper is to report on our normal morphological findings. The histochemical and biochemical background, and corresponding findings in the human eye and changes occurring in glaucoma will be reported later.

MATERIAL AND METHODS

The normal structure of the trabecular meshwork and sclera cannot be satisfactorily studied from autopsy material and enucleated diseased eyes only for especially the ground substance and also other connective tissue elements occurring in this region are changed under these conditions. We therefore used the eyes of laboratory animals and eyes from slaughter-house material.

Eyes of calf, cow, sheep, and young and old rabbits were sectioned meridionally and fixed in 10 per cent formol, Helly's solution, Barany's solution (4), and 4 per cent lead acetate. Dehydration was effected by means of routine ethanol treatment. Paraffin sections 5 μ thick were used.

As lead acetate fixation and Mallory stain finally constituted our principal method we should like to give a detailed description of the procedure. The eyes were sectioned immediately after death and submersed for 48 hours in lead acetate; they were then kept for 24 hours in running water and for the same time in the following substances: 80 and 96 per cent ethanol, absolute alcohol, xylol and paraffin. After treatment in xylol, absolute alcohol, 96 per cent ethanol and water the sections were kept four hours in saturated mercuric chloride solution at 58°C and then rinsed in water. After 10 minutes in 1 per cent acid fuchsin the slides were submersed for 30 minutes in Mallory's solution (anilin blue 0.5 g, orange G 2, 1 per cent phosphor molybdenic acid 100 g, all made by Merck). The differentiation took place in 96 per cent ethanol and finally, after treatment in absolute alcohol and xylol, the slides were embedded i Canada balsam.

FINDINGS

In the sclera proper the bundles formed by the collagenous fibres change their appearance at the border between the cornea and the sclera. In scleras fixed in formol and Helly's solution the corneal, more straight bundles become tortuous and at the same time an extremely complicated system of criss-crossing loops originates. A curved course of fibres was visible especially in calf and cow.

In addition to these fibres, we observed transverse bundles which are easy to identify after lead acetate and sublimate treat-

ment (Figs. 1 and 2). There thus originates spongy structure which is best developed near the cornea and extends in calf from the limbus to the height of the posterior edge of the ciliary body. The structure is, however, the same even in the scleral area as a whole although the fibres are straighter and more regular, giving the spongy structure as a whole a much more regular appearance there (Fig. 3). There is some variation in the appearance of this area in the different animal species examined. The fibres are more tortuous in rabbit and sheep, for instance, than in calf and cow.

On the surface of the scleral bundles we observed fine parallel threads, densely situated, constituting a fairly regular structure. This structure is best-developed in the anterior part of the sclera where the threads are long. There are, however, clear spaces between the bundles and their threads. A similar structure occurs in the posterior part of the sclera, but the spaces here are larger. A bundle containing these threads often resembles a feather or a lamp brush. The threads are easily observed in Helly-fixed and Mallory-stained specimens.

In lead-acetate-fixed and in mercuric chloride-treated and Mallory-stained slides we observed in all the eyes studied an intensely red-stained substance, mainly localised in the sclera proper.

In the anterior part of the sclera, especially in the calf, the red substance is very striking (Fig. 4a). The episcleral tissue contains no red substance.

The collagenous fibres run from cornea to sclera without any visible demarcation. The border, however, it excellently distinguished by the red ground substance (Fig. 4a).

Of special interest is the localisation of the red ground substance in the neighbourhood of Schlemm's canal or more exactly its venous substitute. The calf eye, with its high concentration of red ground substance in the anterior part of the sclera, provides a satisfactory object for study of this phenomenon. On the outer side of Schlemm's canal there is an uniformly staining substance. On the other hand the pore tissue of the internal wall contains a similar substance only sporadically; this, however, stained reddish-brown yellow with the usual technique. The side inward from Schlemm's canal consequently had no uniform ground substance of this type. There were spaces which, as in the uveal network, did not contain this red or reddish ground substance.

In the posterior part of the sclera also there is an easily stainable red ground substance which is more prominent in the cow than in the calf. In rabbit and sheep, too, this posterior ground substance is very prominent.

With the lead acetate—mercuri cchloride—Mallory technique the collagenous bundles stain an intense red (Fig. 5). We have assumed that this is connected with the collagenous bundles with their threads, the spongiform system which might well extend from the skeleton through the ground substance. Comparison of eyes fixed by different methods for the size of the bundles in the anterior part of the sclera showed these to be much thicker in lead acetate than in Helly fixation. There the collagenous fibres with their feather-like systems and ground substances had formed a fairly firm meshwork (Fig. 5).

Topographical differences were observed in the size of the bundles in the sclera. The thickest bundles were found in the middle of the anterior part of the sclera. The lead acetate technique showed up the smallest bundles and also the smallest channels in the most anterior part of the sclera, near the cornea (Fig. 4b).

Granules occur sporadically in the ground substance showing that it is not altogether homogeneous (Fig. 6) and may display physico-chemical variations. These granules are considerably smaller than pigment grains.

In the areas where the red ground substance was found pigment and chromatophores were of regular appearance. Also in young calves and rabbits a good many pigment cells were observed in the sclera proper. The highest concentration of these cells was found in the deeper part of the anterior sclera near Schlemm's canal. Only a little part was found to be blood pigment. In the lamina fusca, of course, numerous pigment cells were found.

DISCUSSION

The bundles of the connective tissue of the sclera and the special arrangement of a spongiform structure filled with easily stainable ground substance provide good protection for the sensitive inner parts of the eye.

The morphological structure of the sclera seems to us to have another important function too. The gel-like ground substance might constitute a resistance to the aqueous outflow from the eye.

Besides the different human collector channels, including the aqueous veins (1, 12, 2[1], 17), which lead from Schlemm's canal to episcleral veins, there are at least two other possible routes for the outflow. The first is via the spaces between the connective tissue bundles. These spaces intercommunicate and make up some kind of channel network. This system of channels is obviously most effective in the anterior part of the sclera where there are curved bundles. Becker and Osterhage also found curved bundles there by tearing the scleras to lamels. According to Ashton (2[1]) the majority of the collector channels anastomose almost immediately after leaving Schlemm's canal to form the deep scleral plexus. In this region the red ground substance stains well and the spaces between the »feathers» are narrow.

Ashton (2[1]) emphasised that collective channels are varicose and that aqueous veins have no histological characteristics and are not specialized structures. Such properties support the assumption that these channels are similar to the channels originating between the feathers. The diameter of the spaces varies with the changes obviously occurring in the gel condition of the ground substance. To this is attributable also the fact that *e.g.* the calf may display variations in the topographical occurrence of spongiform tissue and ground substance.

The second possible route of aqueous outflow is the diffusion of humour through the ground substance in gel condition. The sclera is assumed by Jasinski to be a water reservoir for the surrounding tissues and to be capable of influencing the intraocular pressure in this way. The cattle sclera quilled more in the anterior than in the posterior part, as stated by Nakamura who assumed this to depend on a higher concentration of ground substance in the anterior part. This assumption agrees with our own observations made on the basis of red ground substance. On the other hand is a swelling of connective tissue bundles under the fixation used also to take into consideration. When collagenous fibers and connective tissue bundles in the episcleral tissue and in other parts of the eye did not stained as in the sclera proper, the swelling of this kind could not be the right explanation of the red staining.

Aqueous diffusion through the ground substance is dependent on the physico-chemical status of the ground substance. A hyalu-
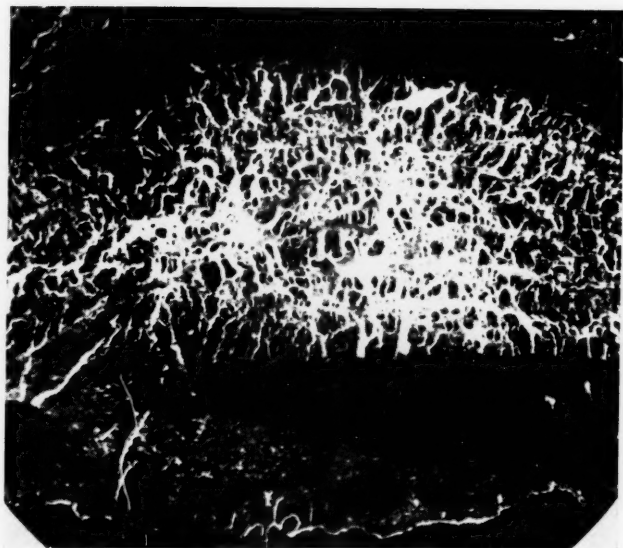
Fig. 1. — Negative of the anterior part of a calf sclera, demonstrating the spongiform structure which is assumed to form a skeleton for the ground substance. Lead acetate-mercuric chloride-Mallory. Magnification 30 ×.
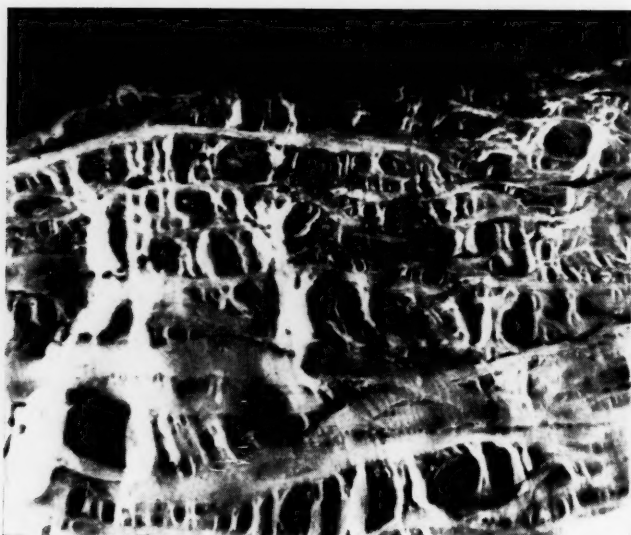
Fig. 2. — Negative of the anterior part of the sclera of a calf, demonstrating
the spongiform structure and the arrangement of »bundles». Note the cross-
striations in the larger bundles. Lead acetate-mercuric chloride Mallory.
Magnification 675 ×.
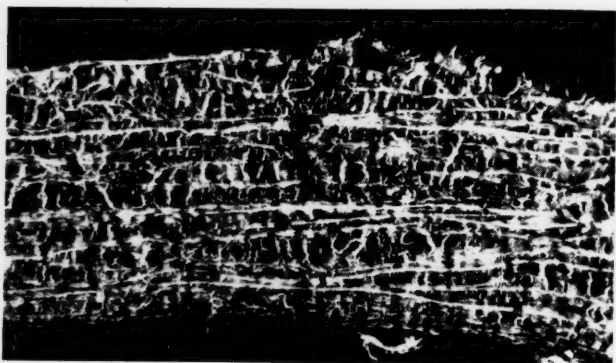
Fig. 3. — Negative of the posterior part of a calf sclera with a more uniform
arrangement of the spongiform structure. Lead acetate-mercuric chloride-
Mallory. Magnification 40 ×.

Fig. 4a. — The red ground substance in the anterior part of a calf sclera. Cornea to the right, and a part of the uvea is seen in the lower right corner. Lead acetate-mercuric chloride-Mallory. Magnification 150 ×.
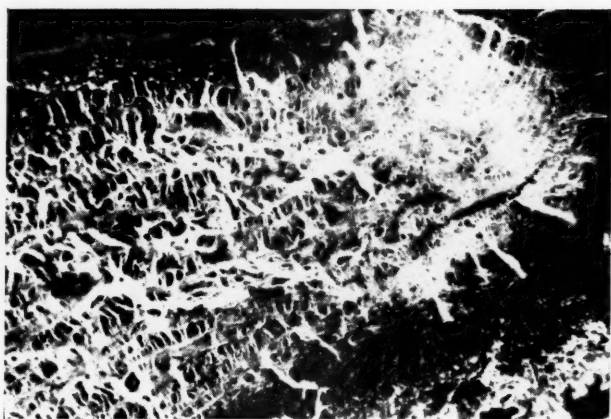
Fig. 4b. — Negative of 4a showing the spongiform skeleton. Magnification 150 ×.

Fig. 5. — The red ground aubstance and the red stained bundles of the anterior part of a calf sclera near the canal of Schlemm. Lead acetate-mercuric chloride-Mallory. Magnification 400 ×.

Fig. 6. — Microphotogram of the same area as Fig. 5 showing granules occurring in the ground substance. Lead acetate-mercuric chloride-Mallory. Magnification 600 ×.

ronidase-sensitive barrier to the outflow of aqueous humour has been described by Bárány and Scotchbrook. Bárány assumes hyaluronidase to have an effect on the ground substance of the specialized connective tissue form'ng the meshwork of the filtering angle. It is, however, natural to wonder whether both hyaluronidase and other substances in the aqueous humour may affect also the scleral ground substance and thus the barrier of the outflow present in it.

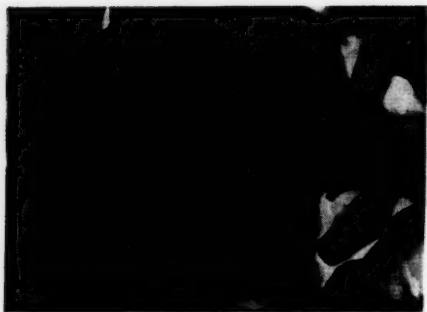The ground substance found in the anterior part of the sclera also gives reason to assume that the barrier here not only serves as a hydrodynamic obstacle for the outflow but that osmotic pressure has a considerable role in the control of intraocular pressure.

Vrabec's interesting report on the occurrence of a metachromatic amorphous substance in the trabecular meshwork, shown up by formol-celodal-cresylviolet technique, with the highest concentration at the tip of the trabecular meshwork close to the cornea and the next highest concentration in the external layers of the meshwork close to the inner surface of the sclera, shows that this part of the chamber angle contains a ground substance. Vrabec is of the opinion that this supports Bárány's hypothesis that the resistance to outflow is localised mainly in the trabecular meshwork.

The next question is whether this is a sufficient barrier since it is assumed that the outflow from the anterior chamber into the canal of Schlemm occurs fairly easily. Dvorak-Theobald, and Ashton, Brini and Smith have proved that Schlemm's canal is really in direct communication with the trabecular meshwork and the anterior chamber. Flocks, however, using tangential sections, could not find such large canals, about 10 μ to 23 μ, as had been described by Dvorak-Theobald. Using microradiographic techniques, Francois, Neetens and Collette injected the anterior chamber of monkeys and human eyes with Thorotrast and Angiopac and observed 1 to 2 μ large pores in the inner wall of Schlemm's canal. Huggert injected rabbit and human eyes chromium phosphate particles and bacteria of various sizes and found the pores to be approximately 1 to 2 μ in size.

From their own experience and the reports of other authors Ashton, Brini and Smith drew the conclusion that open pathways there do not exist in definite endothelial lined tubes but rather as tortuous communications wandering irregularly and obliquely

through the trabecular meshwork like channels through a sponge. This conforms on the whole with the impression we gained of the spongiform structure of the sclera and the occurrence of ground substance in it. We consider it probable that the amorphous substance described by Vrabec in the trabecular meshwork is not the only outflow barrier and that it is probably not the principal barrier. The main barrier is possibly situated in the sclera proper, outside the canal of Schlemm. This assumption is supported by Grant's observation that the trabecular meshwork in some enucleated eyes could be torn away over a quarter to a third of the circumference of the chamber angle without altering the facility of outflow.

The anterior part of sclera proper as far as the ora with its specialized bundles, threads and ground substance we have called the scleral drainage area. Although the posterior part is also of spongiform structure and also contains red ground substance, we are so far not fully convinced of its role as a more important drainage system. Dohlman injected rabbits intravenously with S^{35} labeled sodium sulphate and observed a high uptake evenly throughout the sclera, sometimes especially high in the posterior part. This observation speaks to some extent for the homogeneity of the ground substance of the different parts of the sclera.

The presence of pigment and chromatophores in the scleral drainage area is interesting. Pigment cells were found in both young and old individuals. Our study does not answer the question whether these pigmentcells are localised in the sclera from the outset or whether they have migrated from the uvea. The latter possibility seems acceptable for the pigment cells are localised exactly in the drainage area. Wolff is of the same opinion and assumes that the pigment cells indicate how malignant diseases of the inside of the eye make their way to the outside. We agree, but assume that these cells also show the physiological outflow route. Their local accumulation in pathological conditions perhaps indicates that there is a barrier to outflow in front of them.

The sclera is almost avascular except for the vessels which pass through it to and from the eye. Nor are there as usually assumed any lymphatic vessels in the sclera. The nutrition of the scleral tissue comes from the aqueous in which, outside the canal of Schlemm, blood is mixed as pointed out by Ashton (2^1) — both from the internal

ocular venous system, via the emissary veins from the ciliary muscle plexus, and from the external ocular venous system via the episcleral and conjunctival veins. The spongiform structure and above-mentioned function as a barrier to the aqueous outflow explain why the vessels are not necessary.

SUMMARY

The sclera of calf, cow, sheep and rabbit was studied histologically.

In the lead acetate fixed and mercuric chloride treated and Mallory stained slides a spongiform structure and a quite easily stainable red ground substance was observed in the anterior part of the sclera. A similar spongiform structure, but more uniform, and the ground substance too, appear in the posterior part of the sclera. This construction of the sclera is assumed to constitute a good protection for the sensitive inner part of the eye and to act as a barrier to aqueous outflow and in this way control the intraocular pressure.

REFERENCES

1. Ascher, K. W.: Am. J. Ophth. 1942:*25:*1174.
2. Ashton, N.: Glaucoma, A Symposium Organized by the Council for the International Organizations of Medical Sciences. — Establ. under the joint auspices of UNESCO and WHO, Ed. by S. Duke-Elder. Blackwell, Oxford, pp. 13—22[1], 18[2], 298[3], 1955.
3. Ashton, N., Brini, A., and Smith, R.: Brit. J. Ophth. 1956:*40:*257.
4. Bárány, E.: Glaucoma: A Symposium. Blackwell, Oxford, 1955, 91—104.
5. Bárány, E., and Scotchbrook, S.: Acta physiol. Scandinav. 1954: *30:*240.
6. Becker, H., and Osterhage, K. H.: Ztschr. ges. Anat. 1933:*101:*294.
7. Dohlman, C-H.: Acta ophth. 1957:*35:*115.
8. Duke-Elder, S.: Glaucoma: A Symposium, Blackwell, Oxford. 1955 p. 308.
9. Dvorak-Theobald, G.: Am. J. Ophth. 1955:*39:*65, Pt. II.
10. Flocks, M.: A.M.A. Arch. Ophth. 1956:*56:*708.
11. François, J., Neetens, A. and Collette, J. M.: Am. J. Ophth. 1955:*40:*491.
12. Goldmann, H.: Ophthalmologica, 1946:*112:*344.
13. Grant, W. M.: A.M.A. Arch. Ophth. 1955:*54:*245.

14. Huggert, A.: Acta ophth. 1955:33:271.
15. Jasinski, M.: Klin. oczna, 1933:11:173.
16. Nakamura: sit. by v. Lauber, H., in Handbuch der mikroskopischen Anatomie des Menschen III: 2, Haut und Sinnesorgane. 2. Auge. Springer, Berlin 1936.
17. Thomassen, T. L., and Bakken, K.: Acta ophth. 1951:29:257.
18. Vrabec, F.: Brit. J. Ophth. 1957:41:20.
19. Wolff, E.: The Anatomy of the Eye and Orbit, Lewis & Co. Ltd. London 1948.

EFFECTS OF RESERPINE AND CHLORPROMAZINE ON THE SHAY RAT

by

T. O. KEYRILÄINEN, J. L. KALLIOMÄKI and M. GRÖNROOS

Several investigators have found that reserpine increases acid gastric secretion both in man and in test animals (3, 21, 4, 7, 10, 13, 20, 8, 23, 19). Clinical observation has also revealed not only the activation of old peptic ulcers during reserpine treatment but also the occurrence of acute ulcers (13, 15, 16, 26). Rider (22) on the other hand, has found that a prolonged reserpine treatment (4 × 0.25 mg a day) has no apparent effect on the secretory conditions of the stomach, and recommends it for the treatment of peptic ulcers because of its tranquillizing effects. Reserpine has not been found to influence the secretion of uropepsin (11).

According to Haverbock *et al.* (13), chlorpromazine decreases the volume of gastric secretion but does not significantly change the free acidity. Kirsner and Ford (19) noted a decrease not only in the volume but in the secretion of HCl as well. Chlorpromazine has also been found — contrary to reserpine — to reduce intestinal motility (9). It has rendered favourable results in the treatment of peptic ulcers, too (2, 12).

The purpose of the present study is to throw light on the effects of reserpine and chlorpromazine on Shay rats (24) in view of ulcer production and secretory conditions.

MATERIAL AND METHODS

The series consisted of 73 male albino rats weighing about 180—220 g. Twenty-five rats belonged to chlorpromazine series, 20 to reserpine series, and 28 to control series. The animals were fasted for 72 hours before the operation in individual cages with wide wire mesh bottoms. Water was allowed *ad libitum* before the *operation*, but witheld after it. All the animals were given subcutaneously 3—4 ml of physiological solution of NaCl 13 hours after operation.

The rats were anaesthetized with aether. The abdomen was opened by a short midline incision extending downward from the xiphoid process, a ligature of linen thread placed at the pyloric sphincter and the incision closed with thread sutures. The animals were killed under light aether anaesthesia 21 to 23 hours after tying off the pylorus. The oesophagus was ligated and stomach removed. A small lit was made on the greater curvature for the collection of the gastric juice. Finally the stomach was cut along whole length of the greater curvature and stretched out on card-board.

As a criterion of the *degree of ulceration* we used the size of ulcers as shown below (18):

```
Class I    Diameter of ulcer 0—2  mm = score  1
  »   II      »       »    »  2— 5  »  =    »    5
  »   III     »       »    »  5—10  »  =    »   10
  »   IV      »       »    »     10  »  =    »   20
Perforation                          =    »   20
```

With this scoring system we obtained for each animal an »*Ulcer Index*» (UI) that gave a satisfactory objective evaluation of ulcerations. The UI of a single rat is the sum of the scores for each ulcer.

After centrifugation to remove cellular debris, *the gastric juice* was measured and the pH determined with glass electrode. Total acidity and free HCL were titrated with 0,01 N—NaOH, with thymolblue as indicator. The peptic activity and the relative peptic concentration (= peptic activity after the elimination of inhibitory substances by dilution with 0.1 N—HCl) in Mett units were estimated by the digestion of egg white (Mett's tubes), as described by Nierenstein and Schiff (14).

Half of the rats in the chlorpromazine series received 0.7 mg chlorpromazine/100 g body weight during 13 hours after the operation; the other half was given the same amount but 12 and 17 hours after the operation. The injections were given subcutaneously.

One half of the rats in the reserpine group were given subcutaneously 0.07 mg reserpine /100 g body weight when operated and 13 hours after the operation. The other half received the first injection of reserpine 7 hours before and the second 13 hours after the operation.

TABLE 1

EFFECTS OF RESERPINE AND CHLORPROMAZINE ON THE SHAY RATS

Group	No. of Rats	No. of Ulcers in Different Ulcer Classes				No of perf.	UI	Secretion ml/100 g/hr	pH	Free HCl /100 ml	Total Acidity /100 ml	Peptic Activity in Mett Units	Relative Peptic Conc. in Mett Units
		I	II	III	IV								
Reserpine	20	41	15	4	3	4	14.8	0.30	1.40	46	97	24	452
Chlor-promazine	25	39	7	2	1	1	5.4	0.30	1.44	41	80	26	384
Control	28	100	22	3	2	1	10.7	0.33	1.32	48	88	26	362

RESULTS

Since there were no significant differences between the groups injected at various times in any series, the whole material is brought together in Table 1.

As seen from the table, the mean UI was 14.8 for the reserpine series, 5.4 for the chlorpromazine series, and 10.7 for the controls. The number of the most serious ulcer formations in the reserpine series is worthy of particular notice. Though the series was the smallest there appeared 4 perforations and 3 IV class ulcers in it whereas the respective figures were $1 + 1$ for the chlorpromazine series and $1 + 2$ for the control group.

The volume of gastric secretion has been calculated per one hour for 100 g body weight. As seen from the table, it was practically the same in all groups.

When examining the pH's of the different groups, no significant difference can be noted between the controls and the reserpine series (a difference of 0.07 ± 0.054); whereas in the chlorpromazine series the pH is somewhat higher than in the controls (a difference of 0.11 ± 0.054). When estimating the pH obtained for the reserpine group, it should perhaps be taken into account that in most cases the gastric juice was perceivably hemorrhagic and so somewhat neutralized.

Free HCl was the same in all three series. As to total acidity, it was higher in the reserpine group than in the chlorpromazine one (a difference of 17.0 ± 6.2), the control series being there between. The actual peptic activity values were identical in all groups; whereas relative peptic concentration was higher than normal in the reserpine group (a difference of 90.0 ± 33.0), there was no deviation from the normal in the chlorpromazine group in this respect.

Our results seem to suggest that reserpine increases ulcer production in Shay rats; the augmented ulcer production is also accompanied by somewhat increased total acidity and elevated relative peptic concentration of gastric juice. Chlorpromazine decreases ulcer production; this is accompanied by a gastric juice which is slightly less acid than normal but with a normal peptic activity.

DISCUSSION

By means of anticholinergic products it is possible to block the parasympathicomimetic effects of reserpine (21); according to earlier investigations reserpine evokes gastric secretion in Heidenhain's pouch and in those of gastric fistulas, which is not dependent upon intact vagal mechanism or upon adrenocortical stimulation; all these observations seem to suggest that reserpine would have a direct effect on the parasympathetic parietal ganglions of the stomach. When evaluating the results obtained in the present investigation, the direct depressant effect produced by reserpine on the hypothalamus has to be considered in addition to its direct effect on the parasympathetic ganglions of the gastric wall, the former of which produces peripheral effects similar to those resulting from the stimulation of the parasympathetic nervous system (6).

According to Anad et al. (1) reserpine affects the hypothalamus directly in two ways: it depresses the sympathetic centers and facilitates the parasympathetic ones. Shay et al. (25) have shown that, as regards the Shay operated rats, there exists a direct relationship between the extent of ulceration and the acid-pepsin content of the gastric juice; consequently, as an explanation to our results might be suggested that reserpine has either a central or direct parietal, or a combined increasing effect on the acid-

pepsin secretion of the stomach in Shay rats and is, then, responsible for the increased ulcer production as well.

In our series chlorpromazine reduced ulcer production without any convincing differences in the secretory conditions of the stomach being observable in comparison with the controls (the pH was, unconvincingly, somewhat higher than that of the control group). Chlorpromazine induces a much stronger depressant action on the increased functional activity of the hypothalamus than reserpine does (5). In a region controlled by the peripheric nerves of the vegetative nervous system, its effects are mostly adrenolytic, and not sympathicolytic or parasympathicolytic ones (17, 5). Probably it also lowes metabolism (5). It seems that gastric secretory conditions would not be the only causative factor of ulcer formation in Shay rats, but that ulceration would be due to some other influencing factor as well. Chlorpromazine, by way of the central nervous system in the first place, might reduce the influence of this factor and so decrease ulcer production in spite of the fact that the secretion of the gastric juice would have remained approximately unchanged.

SUMMARY

The writers studied the effects of parenteral reserpine and chlorpromazine on the gastric secretion and the ulcer formation in Shay operated rats. The following results were obtained:

1) Reserpine augments ulcer production and it probably increases total acidity and relative peptic concentration.

2) Chlorpromazine reduces ulcer production, the secretory conditions remaining well within normal limits.

REFERENCES

1. ANAND, B. K., DUA, S., and MALHOTRA, C. L.: J. Pharmacol. 1957:
    *12:*8.
2. ASHER, L. M.: J. Amer. Med. Ass., 1956:*160:*1281.
3. BARRET, W. E., RUTLEDGE, R. A., and ROGIE, B.: Fed. Proc. 1954:
    *13:*334.
4. BARRET, W. E., BLUMMER, A. J., EARL, A. E., and ROGIE, B.: J. Phar-
    macol. Exper. Ther. 1955:*113:*3.
5. BEAN, J. W.: Amer. J. Physiol. 1957:*187:*389.
6. BEIN, H. J.: Experientia 1953:*9:*107.
7. BEMAN, F. M., KNOLL, H. C., and DE LOR, C. J.: Clin. Res. Proc.
    1955:*3:*131.
8. BOM, F., FRIDERICHSEN, T., and ROTHE JENSEN, A.: Ugeskr. Laeg.
    1956:*118:*329.
9. CELICE, J., PORCHER, P., PLAS, F., HELIE, J., and PELTIER, A.:
    Thérapie 1955:*10:*30 (quoted by Exc. Med. VI 1956:*10:*1164).
10. CLARK, M. L., and SCHNEIDER, E. M.: Gastroenterology 1955:*29:*877.
11. GUMMINS, J. F., and BALFOUR, D. C.: J. Amer. Med. Ass. 1956:*161:*864.
12. GIACOMASSO, P. P., PAGANELLI, F., and DePALMA, G.: Gazz. Med.
    ital. 1956:*115:*146.
13. HAVERBOCK, B. J., HEVENSON, T. D., SJOERDSMA, A., and TERRY,
    L. L.: Amer. J. Med. Sc. 1955:*230:*601.
14. HAWK, P. B., OSER, B. L., and SUMMERSON, W. H.: Practical Physio-
    logical Chemistry. 1949.
15. HOLLISTER, L. E., KRIEGER, G. E., KRINGEL, A., and ROBERTS,
    R. H.: ANN. N. Y. Acad. Sc. 1955:*61:*92.
16. HUSSAR, A. E., and BRUNO, E.: Gastroenterology 1956:*31:*500.
17. JOURDAN, F., DUCHENNE-MARULLAZ, and BOISSIER: Arch. Internat.
    pharmacodyn. thér. 1955:*101:*253 (quoted by Exc. Med. VI 1956:
    *10:*782).
18. KEYRILÄINEN, T. O., and PAASONEN, M. K.: Acta pharmacol. et
    toxicol. 1957:*13:*22.
19. KIRSNER, J. B., and FORD, H.: Arch. Int. Med. 1957:*99:*390.
20. KROGSGAARD, A. R.: Nord. Med. 1955:*54:*1851.
21. PLUMMER, A. J., EARL, A., SCHNEIDER, J. A., TRAPOLD, J., and
    BARRET, W.: Ann. N. Y. Acad. Sc. 1954:*59:*8.
22. RIDER, J. A.: Proc. Soc. Exper. Biol. Med. 1955:*90:*636.
23. SCHNEIDER, E. M., and CLARK, M. L.: Amer. J. Digest. Dis. 1956:*1:*22.
24. SHAY, H., KOMAROV, S. A., FELS, S. S., MERAN E, D., GRUENSTEIN,
    M., and SIPLET, M.: Gastroenterology 1945:*5:*43.
25. SHAY, H., KOMAROV, S. A., SIPLET, M., and GRUENSTEIN, M.: Amer.
    J. Digest. Dis. 1947:*14:*99.
26. WOFFORD, J. D., and GUMMINGS, A. J.: New England J. Med. 1956:
    *225:*1193.

ACETAZOLAMIDE AND THE TUBULAR TRANSPORT OF PHENOLSULPHONEPHTHALEIN

by

J. L. KALLIOMÄKI and A. KASANEN

INTRODUCTION

Acetazolamide inhibits the activity of carbonic anhydrase in the kidneys also (Friedberg *et al.* 1953), thus causing an increased excretion of HCO_3^-, Na^+, K^+ and H_2O. The NH_4^+ ion production in tubulus cells decreases or ceases, possibly because of diminished availability of H^+ ions to unite with NH_2. By the action of acetazolamide the urine thus becomes alkaline and a mild renal tubular acidosis develops.

Phenolsulphonephthalein (p.s.p.) is eliminated chiefly by tubular excretion, and for this reason it has been used for over 40 years as an aid in the examination of renal function (Rowntree & Geraghty 1912). These investigators reported that the clinical diuretics in use at that time had no effect on the p.s.p. excretion. A greatly diminished diuresis, on the contrary, may decrease its excretion (Lundsgaard & Møller 1926).

The object of the present work was to study the effect that the inhibition of carbonic anhydrase by acetazolamide has on the tubular transport of p.s.p.

MATERIAL AND METHODS

The acetazolamide series comprised 20 patients hospitalised in the medical clinic. Persons with cardiac failure and febrile patients were excluded.

P.s.p. was injected intramuscularly and the patients ingested 4 dl of water before the test. The percentage of p.s.p. excreted was determined 1 hr. 10 min. and 2 hrs. 10 min. after injection.

Acetazolamide (Diamox[R]) was given at 07 o'clock and the p.s.p. injection at 09 o'clock. For control, increased diuresis was produced in 10 patients by giving them at 07 o'clock 0.14 gm of mercurial diuretic (Thiometrin[R]) instead of acetazolamide.

RESULTS

The effect of acetazolamide on p.s.p. excretion is shown in table 1 and that of mercurial diuretic in table 2.

TABLE 1

EFFECT OF A CARBONIC ANHYDRASE INHIBITOR (DIAMOX[R]) ON THE EXCRE-
TION OF PHENOLSULPHONEPHTHALEIN IN TWO HOURS TEST

Case No.	Excretion of Phenolsulphonephthalein					
	Before Acetazolamide			Two Hours after Administration of Acetazolamide		
	1 st hr. %	2nd hr. %	Total %/2hr.	1 st hr. %	2nd hr. %	Total %/2hr.
1	22	19	41	—	—	69
2	14	37	51	73	8	81
3	12	29	41	21	57	78
4	37	19	56	44	23	67
5	50	50	100	58	28	86
6	11	17	28	8	37	45
7	20	20	40	24	38	62
8	26	36	62	25	66	91
9	57	14	71	43	17	60
10	50	23	73	22	27	49
11	30	32	62	29	58	67
12	19	21	40	43	20	63
13	48	15	63	27	49	76
14	73	27	100	53	23	76
15	47	25	72	69	28	97
16	26	35	61	28	47	75
17	33	1	34	22	22	44
18	41	25	66	44	39	83
19	60	21	81	41	20	61
20	47	27	74	50	10	60
$\bar{x} \pm \varepsilon \bar{x}$ σ	36	24	60±4.2 19	38	32	70±3.3 14

TABLE 2

EFFECT OF A MERCURIAL DIURETIC (THIOMERIN[R]) ON THE EXCRETION OF PHENOL-
SULPHONEPHTHALEIN IN TWO HOURS TEST

| Case No. | Excretion of Phenolsulphonephthalein | | | | | |
| | Before Mercurial | | | Two Hours after Administration of mercurial | | |
	1 st hr. %	2nd hr. %	Total %/2hr.	1 st hr. %	2nd hr. %	Total %/2hr.
1	56	23	79	38	33	71
2	18	37	55	22	34	56
3	14	13	27	12	25	37
4	19	35	54	30	18	48
5	4	12	16	4	8	12
6	31	15	46	41	40	81
7	44	27	71	28	31	59
8	51	15	66	53	20	73
9	40	26	66	16	52	68
10	60	17	77	65	14	79
×	34	22	56	31	27	58

It is seen from table 1 that acetazolamide produced a rise in the p.s.p. excretion in 14 of the 20 patients. The mean difference in 2 hrs. was 10 ± 4.29 per cent. Regarding the 6 patients in whom acetazolamide did not produce increased p.s.p. excretion we observe that already the primary p.s.p. excretion of these patients was very high (100, 71, 73, 100, 81 and 74 per cent). The initial values of 100 per cent were already abnormally high. As is seen from table 2, the mercurial diuretic did not have a uniform effect on p.s.p. excretion.

DISCUSSION

It is very difficult to state the reason for the highly probable promoting effect of acetazolamide on the tubular transport of p.s.p. The increased diuresis does not provide an explanation, since the same effect would have been expected also in the patients receiving mercurial diuretic, and since also other diuretics have not been found to influence the p.s.p. excretion, as was already mentioned above. It is known that substances of the carinamide type inhibit p.s.p. excretion and Beyer *et al.* (1950) are of the opinion that compounds of this type block the ability of this definitive

system to utilise energy from phosphorolysis of energy-rich phosphates for a conjugate reaction essential for ultimate excretion of p.s.p. The possibility that acetazolamide would have the opposite effect in this respect must in our opinion be taken inte consideration. The increased production of H^+ ions in the tubular cells due to acetazolamide must also be held in mind in considering the reasons for the increased p.s.p. excretion produced by acetazolamide. Mercurial diuretics inhibit, for example, the effect of succinic dehydrogenase in tubulus cells (Mustakallio & Telkkä 1953) and they have no effect on the p.s.p. excretion. It is also possible that a small proportion of p.s.p. would after all be reabsorbed in the tubules and that acetazolamide would inhibit this event, in similarity to its inhibition of Na^+ reabsorption. However, the effect would have to be specific, since mercurial diuretics also influence the reabsorption events.

It therefore can only be said that acetazolamide promotes the tubular transport of p.s.p. in some manner which cannot be explained on basis of the results of the present study.

SUMMARY

Acetazolamide increased the excretion of phenolsulphonephthalein in 14 out of 20 patients in a 2-hour test. In the 6 patients whose excretion did not increase, the primary excretion already was high (over 71 per cent in 2 hrs.). The difference in the mean excretion was 10 ± 4.29 per cent in 2 hrs. In a series of 10 control patients who were given mercurial diuretics instead of acetazolamide no uniform changes were seen in the phenolsulphonephthalein excretion.

REFERENCES

1. BEYER, K. H., WIEBELHAUS, V. D., TILLSON, E. K., RUSSO, H. F., and WILHOYTE, K. M.: Proc. Soc. Exper. Biol. Med. 1950:74:772.
2. FRIEDBERG, C. K., TAYMOR, R., MINOR, J. B., and HALPERN, M.: N. England J. Med. 1953:248:883.
3. LUNDSGAARD, C., and MØLLER, E.: Acta Med. Scand. 1926:63:242.
4. MUSTAKALLIO, K. K., and TELKKÄ, A.: Science 1953:118:320.
5. ROWNTREE, L. G., and GERAGHTY, J. T.: Arch. Int. Med. 1912:234:9.

LACTIC ACID RESPONSE TO MUSCULAR EXERCISE IN RHEUMATOID ARTHRITIS

by

J. L. KALLIOMÄKI, M. PULKKINEN, and P. SAVOLA

When examining intermediary metabolism in patients with rheumatoid arthritis (r.a.) Lövgren (7) observed the occurrence of deviations which might be indicative of disturbed carbohydrate metabolism. In vitro examinations of human synovial membrane have shown that in the rheumatoid tissues very marked increase in glucose utilization and lactate production over the normal values are evident (3). Animal experiments have demonstrated, as well, that in connective tissue cells, both in aerobic and anaerobic conditions, most of the glucose consumed by the cells was converted to lactic acid (4). R.a. is very often attended by severe muscular atrophy and fatigue. Since r.a. reveals indication of general disturbances in carbohydrate metabolism, signs of increased anaerobic glycolysis in rheumatoid connective tissue and symptoms in skeletal muscular system, we consider it deserving to examine the lactic acid production in blood due to relative oxygen lack which has been brought about by bodily exercise.

MATERIAL AND METHODS

The material consists of 11 patients with r.a. (10 men and 1 woman). All of them were bodily trained and most patients were manual labourers. The age varied from 30 to 50 years and in respect to the stage of the disease the patients were distributed as follows:
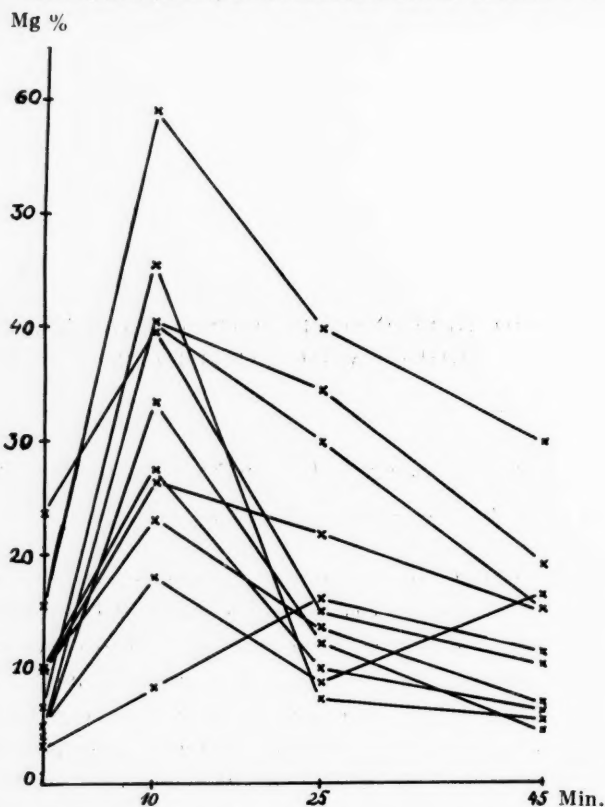
Fig. 1. — Lactic acid response to muscular exercise in cases with rheumatoid arthritis.

I stage — none, II stage — 3 cases, III stage — 6 cases, and IV stage — 2 cases. ESR varied between 12 and 90 mm/hour (mean 50 mm/hour). None of the patients was under steroid therapy.

Since physical training considerably affects the results achieved in this kind of exercise test (6), it was the intention of the writers to select the controls (9 men and 1 woman) so as to correspond as closely as possible to the original series.

The exercise, which was undergone in postabsorptive stage after 15 minutes of complete rest, was produced by means of a cycle-ergometer. For each patient the exercise was 700 kgm/minute for 10 minutes, that is a total of 7000 kgm in 10 minutes. A complete rest followed the exercise.

Blood samples from the cubital vein were taken without stasis
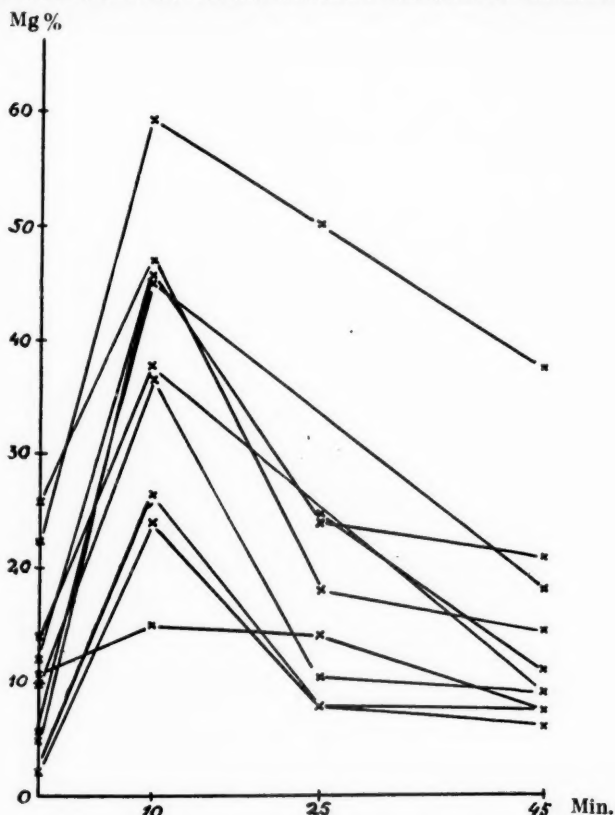
Fig. 2. — Lactic acid response to muscular exercise in control cases.

immediately before and after the exercise, and at 15 and 35 minutes after the completion of the exercise. The whole experiment took, then, 45 min.

Lactic acid was determined from blood as soon as the experiment was over by using Barker and Summerson's (1) method. All the results are based on double determinations.

RESULTS

Figs. 1 and 2 show for each case the lactic acid response of blood to the exercise.

The mean rest-value obtained before the exercise was 8.6 ± 1.8 mg% (SD 5.8) for the r.a. series and 11.0 ± 2.4 mg% (SD 7.5) for the controls. The difference between the rest-values is 2.4 ± 3.0

Immediately after the exercise the mean was 32.9 ± 4.2 mg%
for the r.a. group and 39.3 ± 4.0 mg% for the controls with a dif-
ference of 6.4 ± 5.8. Fifteen minutes after the exercise the means
were 19.1 mg% and 20.0 mg% and after 35 minutes 12.7 mg% and
14.1 mg% respectively.

DISCUSSION

The results obtained show quite clearly that the lactic acid
response to muscular exercise in patients with r.a. was almost
identical with that of the controls. It is true that the mean values
were all the time somewhat lower for the r.a. series, but the differ-
ence is statistically insignificant. In this connection it may be
mentioned, however, that examinations of serum lactic dehydro-
genase levels in various disease states have revealed elevated levels
in patients suffering from r.a. (5). This might indicate a greater
speed of pyruvic acid ⇌ lactic acid reaction than in healthy persons.

The liver plays a very important role in the removal of the excess
lactic acid from the blood (2). Since the lactic acid values became
normal just as quickly in the r.a. group as in the control series, our
material does not reveal any disturbances in this mechanism in
patients with r.a.

Neither does is seem likely that the disturbed anaerobic glycoly-
sis in connection with muscular fatigue or a disturbance in the
removal of the excess lactic acid from the blood would contribute
or give rise to the muscular atrophies and the muscular fatigue
met with in r.a.

SUMMARY

1) Eleven bodily trained patients with rheumatoid arthritis
and 10 similarly trained controls underwent, pedalling a cycleergo-
meter for 10 minutes, an exercise corresponding to 7000 kgm. The
effect of the exercise on blood's lactic acid level was examined.

2) It was found that in patients with rheumatoid arthritis the
lactic acid response to muscular exercise did not differ significantly
from normal.

REFERENCES

1. BARKER, S. B., and SUMMERSON, W. H.: J. Biol. Chem. 1941:*138*:535.
2. CORI, C. F., and CORI, G. T.: J. Biol. Chem. 1929:*81*:389.
3. DINGLE, J. T. M., and THOMAS, D. P. P.: Brit. J. Exp. Path. 1956:*37*:318.
4. HARRIS, H.: Brit. J. Exp. Path. 1956:*37*:512.
5. HSICH, K. M., and BLUMENTHAL, H. T.: Proc. Soc. Exp. Biol. Med. 1956:*91*:626.
6. LINKO, E.: Ann. med. int. Fenniae 1950:*39*:161.
7. LÖVGREN, O.: Acta Med. Scand., Suppl. 163 (1945).

PHENYLBUTAZONE AND STOMACH

EXPERIMENTAL STUDIES ON THE SHAY RAT

by

J. L. KALLIOMÄKI, T. O. KEYRILÄINEN and M. GRÖNROOS

Most clinical publications dealing with phenylbutazone (phb), mention hemorrhages in the gastrointestinal tract and the occurrence or activation of peptic ulcers as complications occasionally met with during phb-treatment. As an example we may mention a series, collected from literature (7), which consisted of 1526 patients, of whom 13 were reported to have developed hematemesis and melaena and 17 to have revealed activation of peptic ulcers under phb-treatment. It has been alleged that orally or intramuscularly given phb increases gastric secretion and gastric acidity in man (5, 2). Lambling & Bonfils (6) represent the opposite view. According to them, phb decreases gastric secretion producing hypochlorhydria attended with diffuse gastritis.

Given orally to test-animals, phb has also effected ulceration in the gastric mucosa (1, 3). The writers of the two publications are of the opinion that phb has a direct influence on gastric mucosa. Hillemand and Cocovinis (3) found phb produce submucosal oedemas as well.

The purpose of the present work is to study what effects phb has on ulceration and on the secretory conditions of the stomach in (a.m. Shay) rats with ligated pylorus.

MATERIAL AND METHODS

The whole series consisted of 66 male albino rats weighing 165—250 g. The material was grouped as follows:

Twenty-seven rats received phb at the time of the operation and 12 hours after the ligation. Nine of them were given 10 mg of phb intramuscularly on both occasions, ie. a total of 20 mg during the experiment. The average weight of these animals, when killed, was 208 g. Half a dose, ie. totalling to 10 mg, was given correspondingly to 9 rats. The average weight in this group was 207 g. The third group of 9 animals got, at corresponding points of time, 25 mg of phb solution orally (a total of 50 mg). The average weight was 202 g at the end of the experiment. Ten animals were used as controls; their average weight was 207 g at the completion of the experiment.

For 19 rats the administration of phb was begun 4 days before the ligation of pylorus. Ten of these rats received daily a dose of 15 mg and a dose of 10 mg immediately when operated. All administrations were intramuscular. Nine rats were given the same doses orally. Ten rats, which were used as controls, were kept in similar conditions for 4 days without giving them phb.

In all groups the animals were given 3—4 ml of physiological NaCl-solution subcutaneously 12 hours after the ligation.

The methods used in the examinations are explained in detail in a previous publication by the authors (4).

EXAMINATION OF RESULTS

The results are shown in Tables 1 and 2.

When examining the effects of an acute administration of phb in Shay rats, shown in Table 1, one perceives the greatest deviation between the controls and the group which was given the smallest dose (10 mg in all) intramuscularly. Small ulcerations, 0—2 mm in diameter, were three times as numerous in this group as in the control series; besides, two rats exhibited perforated ulcers, where as none was met in the controls. Only two rats of the group revealed unaffected stomachs when the experiment was over. This amount of phb, then, increased ulceration, yet the amount of secretion, pH, acidity, and pepsin secretion did not differ significantly from those of the controls. Curiously enough, when the intramuscular dose was doubled (20 mg in all), ulcer formation, instead of increasing, dropped even lower than among the controls. In this group only two stomachs were found affected at the end of the experiment.

TABLE 1

THE EFFECTS OF INTRAMUSCULAR OR PERORAL ADMINISTRATIONS OF PHB ON THE SHAY RATS, GIVEN AT THE TIME OF THE OPERATION AND 12 HOURS AFTER IT

Group	No of Rats	Pylorus Ligated hrs	Stomach Wall No. of Ulcers Grouped according to Diameter of Ulcer				No. of Perforations	No. of Unaffected Stomachs	Gastric Juice Secretion ml/100 g hr	pH	Free HCl/100 ml	Total Acidity/100 ml	Peptic Activity in mett Units	Relative Peptic Conc. in mett Units
			0—2 mm	2—5 mm	5—10 mm	>10 mm								
Intramuscular Route 10 mg at the moment of operation and 10 mg 12 hrs after it	9	24	15	1	—	1	—	7/9	0.17	1.6	29	107	15	143
Intramuscular Route 5 mg at the moment of operation and 5 mg 12 hrs after it	9	24	41	3	—	1	2	2/9	0.27	1.5	36	119	17	166
Peroral Route 25 mg at the moment of operation and 25 mg 12 hrs after it	9	24	29	5	1	—	1	3/9	0.26	1.7	38	115	15	115
Control	10	24	14	3	—	—	—	5/10	0.25	1.5	55	107	15	131

TABLE 2

THE EFFECTS OF A FOUR DAYS' INTRAMUSCULAR OR PERORAL PHB-THERAPY ON THE SHAY RATS

Group	No. of Rats	Pylorus Ligated hrs	Stomach Wall							Gastric Juice					
			No. of Ulcers Grouped according to Diameter of Ulcer				No. of Perforations	No. of Unaffected Stomachs		Secretion ml/100 g hr	pH	Free HCl/100 ml	Total Acidity/100 ml	Peptic Activity in Mett Units	Relative Peptic Conc. in Mett Units
			0—2 mm	2—5 mm	5—10 mm	>10 mm									
Intramuscular Route 15 mg daily on 4 days before operation and 10 mg at the moment of operation	3	12	8	2	—	—	—	2/3		0.62	1.2	56	105	19	228
	7	24	111	46	11	1	—	1/7		0.47	1.5	30	80	21	229
Peroral Route 15 mg daily on 4 days before operation and 10 mg at the moment of operation	3	12	2	—	—	—	—	2/3		0.40	1.5	46	122	19	235
	6	24	63	15	4	—	1	1/6		0.36	1.5	39	85	23	245
Control	3	12	—	—	—	—	—	3/3		0.66	1.2	71	113	15	214
	7	24	44	20	2	—	1	1/7		0.43	1.3	55	95	16	259

Furthermore, the secretion values were uniformly lower in this group than in any other series, including the controls as well. This finding is to be considered significant. Acidity and pepsin values did not deviate from normal in this group either. To find an explanation of this paradoxical discrepancy between the effects of a large and a smaller dose of phb, is not easy, nor is our material sufficient grounds for such an explanation. A theoretical possibility would naturally be that the submucosal oedema effected by the larger dose was so severe as to decrease the amount of secretion and thus ulcer formation too. This discrepancy may partly be responsible for the contradictory opinions in literature about the effects of phb.

Phb, when given through the mouth to rats with ligated pylorus, doubled ulcer formation as compared with the controls. Secretory conditions did not differ significantly from normal. Ulceration was, then, somewhat lower in the »peroral series» than in the group which received the smaller intramuscular dose.

When examining the effects of a prolonged administration of phb in Shay rats, shown in Table 2, one finds ulcer formation correspond to the one noticed in the short-time administration of phb. In this series, 3 animals of each group were killed 12 hours after the ligation. The controls revealed only intact stomachs, whereas 2 of the 6 rats that had received phb, exhibited ulceration. The average values, shown in Table 2, which represent secretory conditions, do not differ significantly from those of the control group.

On the basis of our results it may be concluded that both intramuscularly and orally administered phb increases ulcer formation in Shay rats without any significant changes in the secretory conditions of the stomach being observable. Large intramuscular doses may, however, decrease the amount of secretion and thus even decrease ulcer formation during these experimental conditions.

SUMMARY

1. The writers studied the effects of an acute and a prolonged administration of phb on the Shay rats.

2. Both intramuscular and peroral administration increased

ulcer formation without any significant changes taking place in secretory conditions.

3. Large intramuscular doses of phb led to a paradoxical decrease in secretion and also to a decreased ulcer formation.

Acknowledgements

REFERENCES

1. ARON, E., LEFREIN, H., and JOBARD, P.: Arch. mal. app. digest. 1956: *45*:496.
2. BELLOMO, F.: J.A.M.A. 1956:*161*:173.
3. HILLEMAND, P., and COCOVINIS, ?.: Arch. mal. app. digest. 1956:*45*:313.
4. KEYRILÄINEN, T. O., KALLIOMÄKI, J. L., and GRÖNROOS, M.: Ann. med. exp. biol. Fenniae, in press.
5. KIRSNER, J. B., and FORD, H.: Gastroenterology 1955:*29*:1.
6. LAMBLING, A., and BONFILS, S.: Rev. méd. franç. 1955:*36*:60.
7. LEONARD, J. C.: Brit. Med. J. 1953:*1*:1311.

VARIATION IN THE COAGULASE ACTIVITY OF FRESHLY ISOLATED STRAINS OF STAPHYLOCOCCI AND THEIR CAPACITY TO GROW IN NORMAL HUMAN SERUM

by

N. OKER-BLOM, T. S. LINDHOLM and IRJA ROUHIA

The capacity to coagulate plasma has since Loeb (3) been regarded as one of the best criteria of the pathogenicity of *Micrococcus pyogenes var aureus*. Smith, Lominsky and Morrison (5), however, by testing some laboratory strains of staphylococci, have shown that there exists a great variation in their capacity to coagulate plasma. Thus, in the same culture there may occur strongly coagulase positive R variants and weakly coagulase positive, or even coagulase negative, S variants. The variants are not stable, however, and transformation from one type to the other may be seen, the transformation from the R form to the S form being the most common.

A somewhat similar observation was made also in this laboratory some years ago. It was observed that some freshly isolated coagulase-negative strains of yellow staphylococci turned out to be coagulase positive when retested one or several days later, and a search for similar strains in this and another laboratory (2) showed that they were not at all uncommon.

Thus, if a single coagulase test is used as a criterion for pathogenicity, some staphylococcus strains may be wrongly classified. Recently Ekstedt and Nungester have shown that only coagulase-positive strains, but not the coagulase negative variants, are capable of growing in fresh human serum (1).

An attempt was therefore made to compare the afore-mentioned late coagulase-positive staphylococcus strains with ordinary coagulase positive strains in respect to their capacity to grow in human serum.

The results of this comparison, as well as experience obtained when studying the variation in the coagulase activity of some freshly isolated staphylococcus strains, will be presented in this paper.

MATERIAL AND METHODS

During this work several hundreds of staphylococcal cultures were isolated mainly from lesions of the skin, exudates, expectorates and urine.

The isolations were made on agar containing 5 per cent defibrinated horse blood; subcultures were made either on the same blood agar or on plain agar or in broth. The cultures were incubated for 24 hours at 37°C.

Coagulase Tests. — Human citrated plasma diluted 1 to 10 with saline was distributed into small test tubes in amounts of 1.5 ml. The tubes were inoculated with a loopfull of culture from a blood agar plate. The reaction was read after 3 hours at 37°C, and another reading was made 20 hours later at room temperature. In some cases the reading was made after 24 hours at 37°C. The degree of coagulation was recorded as $+++$ (total coagulation), $++$ (partial coagulation), $+$ (a small coagulum in the bottom of the tube), $\pm$ (positivity doubtful) and — (no traces of coagulation). In every test one tube of uninoculated plasma and one tube with plasma inoculated with a known coagulase-positive strain were included as negative and positive controls, respectively.

Coagulase Titer. — This was estimated according to the method described by Smith, Morrison and Lominsky (5).

Antibacterial Tests. — The tests were performed by a technique on the whole similar to that described by Ekstedt and Nungester (1).

Small test tubes containing 0.9 ml of the serum were inoculated with 0.1 ml of a 10^{-6} dilution of an overnight broth culture of the staphylococci to be tested. The tubes were incubated for 24 hours at $+37°C$. Viable bacteria were counted both in the inoculum and in the serum by making tenfold dilutions in ordinary broth. From

each dilution 1.0 ml was evenly distributed on a blood agar plate and the colonies counted.

Serum from a single donor was used for all the experiments. When the blood had clotted, the serum was separated and stored at —15°C.

<center>RESULTS</center>

Among the staphylococci isolated 30 otherwise typical *Staphylococcus aureus* strains were coagulase-negative. These strains were kept on the same blood agar plate at room temperature for 24 to 48 hours or more and retested. Seventeen (17) of these strains, when retested, gave a positive coagulase test 24 hours later, and an additional group of 8 strains were positive when tested after 48 hours at room temperature. Five strains remained coagulase negative.

To obtain an idea of the coagulase activity of different colonies in the same culture, five single colonies from each of 14 blood agar plates streaked with 14 different cultures of *Staphylococcus aureus* were tested. Simultaneously several colonies were scraped together from the same blood plate and the loopfull of bacteria thus obtained tested. As mentioned above, this was the routine method used when freshly isolated cultures were tested for coagulase activity.

The cultures containing only positive colonies (+ to +++) were all positive when tested according to the routine method. A single culture showing only coagulase-negative colonies was coagulase-negative in the routine test. Of four cultures showing both positive and negative colonies a positive coagulase test was obtained according to the routine method only if the positive colonies were in excess.

A similar test was performed on freshly isolated cultures simultaneously with the routine test. In this case 9 different colonies were tested, and the result was read after 3 and 24 hours respectively. These results were very similar to those described in the previous experiment, *i.e.* cultures containing only positive colonies or predominantly positive colonies were positive according to the standard method. In addition, these experiments also showed that three hours' incubation of plasma at 37°C does apparently not, by using this method, reveal all coagulase-positive cultures, and an

additional time of incubation either at room temperature or at 37°C seems to be neccessary.

Neither of these experiments did, however, show whether the change in coagulase activity of the culture depended on a change in coagulase activity of single colonies or simply on the fact that the first test was made on predominantly coagulase-negative colonies and the second or one of the following predominantly on coagulase-positive colonies. For this reason the same colonies were tested several times.

These tests supported the observation made by Smith, Morrison and Lominsky, that the R variants are usually strongly positive in the coagulase test whereas the S variants are usually only weakly positive or negative. When the same colonies were retested, however, some of the coagulase-negative S variants appeared to be weakly coagulase-positive and in some cases even more strongly positive showing an $++$ reaction. R variants tested under equal conditions also showed a change towards stronger coagulase positivity.

Four of the 30 cultures were also tested for their coagulase titer. Three of the strains had changed from coagulase negativity to positivety whereas one remained negative. An initially coagulase-

TABLE 1

COAGULASE TITER OF DIFFERENT STAPHYLOCOCCUS STRAINS

Type of Strains	Strain	Degree of Coagulase Activity Dilution of Culture Fluid							
		1: 2	1: 4	1: 8	1: 16	1: 32	1: 64	1: 128	1 :256
Initially and permanently coagulase positive ..	25	$+++$	$+++$	$+++$	$+$	$\pm$	$\pm$	$\pm$	$-$
Initially coagulase negative	2	$+$	$+$	$\pm$	$\pm$	$-$	$-$	$-$	$-$
	3	$+$	$\pm$	$\pm$	$-$	$-$	$-$	$-$	$-$
Later coagulase positive	9	$++$	$\pm$	$\pm$		$-$	$-$	$-$	$-$
Initially and permanently coagulase negative	13	$-$	$-$	$-$	$-$	$-$	$-$	$-$	$-$

$+++$ = total coagulation
$++$ = partial coagulation
$+$ = small coagulum in the bottom of the tube
$\pm$ = positivity doubtful
$-$ = no traces of coagulation

TABLE 2

GROWTH OF DIFFERENT STAPHYLOCOCCUS STRAINS IN NORMAL HUMAN SERUM

Type of Strains	Strain	Coagulase Activity	Approximate Number of Bacteria Inoculated	Multiplication Factor [1]
Initially and permanently coagulase positive	4	+ + +	10	5
	6	+ + +	10	5
	7	+ + +	10	4
	8	+ + +	10	5
	9	+ + +	10	6
	10	+ + +	10	5
	17	+ + +	10	6
	18	+ + +	10	7
	1	+ + +	100	6
	2	+ + +	100	4
	3	+ + +	100	5
	5	+ + +	1000	4
Initially coagulase negative Later coagulase positive	2×	+ + +	10	5
	3	+	10	6
	9	+ + +	10	6
	12	+ +	10	6
	15	+	10	5
	17	+ +	10	5
	20	+ +	10	5
	22	+ +	10	6
	20×	+ +	10	6
	1057	+	10	6
	4	+ +	10	5
	1169	+ +	10	6
	2st	+ +	10	6
	1st	+	10	6
Initially and permanently coagulase negative	13	—	10	3
	18	—	10	3
	3st	±	10	5
	4740	—	10	5
Initially and permanently coagulase negative	14	—	10	2
	16	—	10	0—1
	19	—	10	0—1
	21	—	10	0
	22	—	10	0
	23	—	10	0
	24	—	10	0
	25	—	10	0
	26	—	10	0
	12	—	100	0
	13	—	100	0
	15	—	10	0

Staphylococcus Aureus (rows 1–28), *Staphylococcus Albus* (rows 29–40)

[1] Log difference between number of bacteria in inoculum and in serum after incubation.

positive culture was included as a control. The results are shown in table 1. The titer of the control is low, but there is still a great difference between the titer of this culture and that of the cultures which had changed from coagulase negativity to positivity.

When testing the capacity of the different strains to grow in normal human serum it was shown that all the initially and usually strongly coagulase-positive strains multiplied well in the serum (table 2). Of some coagulase-negative typical albus strains only one out of 10 showed some slight multiplication, whereas the others did not grow at all.

The strains which had changed from coagulase negativity to positivity behaved, as expected,like the initially coagulase-positive *Staphylococcus aureus* strains. Of the Staphylococcus aureus strains which did not produce coagulase even after storage and subcultures, however, two grew well and two fairly well in the human serum, showing a multiplication factor of 5 and 3 respectively (table 2). Repeated coagulase tests performed on these strains were negative.

DISCUSSION

The coagulase test is usually performed either by using over-night broth cultures of staphylococci or broth suspensions of colonies from an agar plate. Thus the coagulase test, as it has been performed in these experiments, is probably not the most accurate means to test the coagulase activity of staphylococci. In many textbooks of practical bacteriology, however, this method is recommended, and many diagnostic laboratories use the test in this form. Independent of the suitability of the test, however, there seem to be differences between the coagulase activity of freshly isolated staphylococcus cultures after the first 24 hours of growth compared to the activity 24 to 48 or more hours later. In addition freshly isolated staphylococcus cultures may contain both coagulase negative and coagulase positive variants.

If looked upon from a practical point of view this indicates first, that several colonies should be tested for coagulase activity and, second, that the primary coagulase test, if performed according to the method used here, does not always reveal coagulase-positive staphylococci. This obstacle can probably be overcome by using

the method of subculture of several colonies it broth, because in
this way several types of colonies will be tested and most of the
initially coagulase negative strains will apparently after this sub-
culture be coagulase positive.

The results of the antibacterial tests are for the majority of the
strains in accordance with the results of Ekstedt and Nungester,
i.e. the capacity to grow in normal human serum seems for all
typical strains to depend on the coagulase production of the strains.
An exception are the four otherwise typical but coagulase-negative
Staphylococcus aureus strains which remained coagulase negative
in spite of several subcultures from several colonies. This may of
course depend either on a very low bacterostatic power of the serum
or an inability to demonstrate a minimal coagulase production. But
it may also depend on differences in the strains themselves.

The experiments do not explain the reason for neither the
variation in the coagulase activity of the *Staphylococcus aureus*
strains nor the capacity of some coagulase negative strains to grow
well in normal human serum. They have been presented therefore
merely to call attention to the irregular results obtained with these
tests. It could probably, however, be of some interest to investigate
this questions further, bearing in mind, for instance, the effect of
in vivo acting antibiotic factors.

SUMMARY

The variation in the coagulase activity of some freshly isolated
Staphylococcus aureus strains, as well as their capacity to grow
in normal human serum has been studied.

Twenty-five out of 30 initially coagulase-negative Staphylo-
coccus aureus strains turned out to be coagulase-positive when
retested 24 or 48 hours later, apparently depending on a change
in the coagulase activity of the cultures during cultivation. All these
25 late coagulase-positive strains grew well in normal human serum
like the typical, initially coagulase-positive *Staphylococcus aureus*
strains, but in contrast to typical coagulase-negative *Staphylo-
coccus albus* strains. Of the remaining, otherwise typical, but
apparently permanently coagulase-negative *Staphylococcus aureus*
strains, two grew well in the same serum.

The irregularity of both tests, as well as the practical and theore-
tical implications, are briefly discussed.

REFERENCES

1. Ekstedt, R. D., and Nungester, W. J.: Proc. Soc. Exper. Biol. Med. 1955:89:90.
2. Kosunen, T., and Parmala, M.: An. Med. exp. biol. Fenn. 1956:34:2.
3. Loeb, L.: J. med. Res. 1903:10:407.
4. Lominsky, I., Smith, D. D., and Morrison, R. B.: Nature 1953:71:214.
5. Smith, D. D., Morrison, R. B., and Lominsky, I.: J. Path. Bact. 1952:64:567.